IN VIVO PROPERTIES OF *PSEUDOMONAS AERUGINOSA*: OUTER MEMBRANE COMPONENTS AND THEIR ANTIGENICITY

by

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Submitted for the degree of Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

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This study involved an investigation of bacterial properties *in vivo* with particular reference to the outer membrane (OM) antigens of such bacteria and their recognition by host immunoglobulins during infection. *Pseudomonas aeruginosa* was recovered directly, without subculture, from the infected wounds of burn patients and the bacterial OM antigens analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The results indicated that the *in vivo* bacteria expressed high molecular weight OM proteins not present in cells of the same isolate cultivated in complex media, unless depleted of iron. Immunoblotting techniques further demonstrated recognition of the iron-regulated membrane proteins (IRMPs) by antibodies present in the patient serum and locally in wound tissue fluid, suggesting their potential as vaccine candidates. Differences between lipopolysaccharide (LPS) antigens from *in vivo* and *in vitro* grown bacteria were additionally observed following analysis by SDS-PAGE.

Methods were devised to purify the IRMPs in a form relatively free from LPS. These included detergent extraction to solubilize the proteins and gel filtration procedures to separate the IRMPs from other OM proteins and subsequently to remove LPS.

An investigation was made of the rabbit humoral immune response to *P. aeruginosa* growing as an adherent microcolony on the surface of inert materials (used in medical prostheses) and implanted into the peritoneum. The resulting localized infection was chronic in nature and involved large numbers of bacteria . Electron microscopy studies revealed that the adherent cells were embedded in an extensive glycocalyx partly composed of exopolysaccharide. Crossed immunoelectrophoresis demonstrated that in this biofilm mode of growth the bacteria avoided evoking a strong immune response, perhaps due to masking of key antigens by the glycocalyx. This was in contrast to bacteria in an acute disseminated peritonitis model, which elicited a high titre of antibodies, notably to the LPS antigen. Moreover, antibodies to the IRMPs were produced at an early stage of the disseminated infection. The results may help to explain the persistence of infections associated with medical prostheses such as peritoneal dialysis catheters.

Key Words: *Pseudomonas aeruginosa*, outer membrane proteins, iron, lipopolysaccharide, biofilms.

TO MY MOTHER

WITH LOVE

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

A	ampere
CAPD	continuous ambulatory peritoneal dialysis
CDM	chemically defined media
CF	cystic fibrosis
CIE	crossed immunoelectrophoresis
cm	centimetre
CM	cytoplasmic membrane
°C	degrees centigrade
DMSO	dimethyl sulphoxide
DNA	deoxy-ribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
FCA	Freund's complete adjuvant
-Fe	lacking or depleted of iron
FIA	Freund's incomplete adjuvant
fla	flagella
GLC	gas liquid chromatography
g	gramme
h	hour
IGIV	immune globulin for intravenous use
IM	intramuscular
IP	intraperitoneal
IRMP	iron regulated membrane protein
IV	intravenous
K	kilodaltons
KDO	2-keto 3-deoxy-D-manno-2-octulosonic acid
L	litre
LPS	lipopolysaccharide
M	moles per litre
µg	microgramme
mg	milligramme
MIC	minimum inhibitory concentration
min	minutes
µl	microlitre
ml	millilitre
mm	millimetre
MOPS	morpholinopropane sulphonic acid
NC	nitrocellulose
ng	nanogramme
nm	nanometre
OD	optical density
OM	outer membrane
OMP	outer membrane protein
PG	peptidoglycan
PL	phospholipids
PMN	polymorphonuclear leucocytes
PMSF	phenylmethylsulphonyl fluoride
RNA	ribonucleic acid
s	seconds
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
Tris	tris (hydroxymethyl) amino ethane
TBS	tris buffered saline

TBS/Tween	tris buffered saline with added Tween 20
TEM	transmission electron microscopy
TSB	tryptone soy broth
UTI	urinary tract infection
V	volt
v/v	volume by volume
w/v	weight by volume

1. Introduction

1.1. Factors affecting bacterial properties in vitro and in vivo.

The prevailing environment exerts a profound influence on the properties of bacteria both in relation to the composition of their cell envelope (Ellwood and Tempest, 1972; Holme, 1972; Brown and Williams 1985, a and b), and to their mode of growth as mobile, planktonic cells or glycocalyx-enclosed adherent microcolonies (Costerton *et al*, 1979; Brown and Williams, 1985a; Costerton *et al*, 1985; Costerton *et al*, 1987). The ability of a microorganism to modify and adapt to its surroundings is clearly advantageous in terms of survival and may be important in determining bacterial pathogenicity (Smith, 1977; Costerton *et al*, 1979). *In vitro* studies have given a clear insight into the flexibility of microorganisms particularly regarding phenotypic variation in cell envelope characteristics induced by changes in growth rate (Gilbert and Brown, 1978 and 1980; Taylor, 1984) and nutrient limitation (Ellwood and Tempest, 1972; Holme, 1972; Brown and Williams, 1985a). In turn, some of these factors may correlate with altered bacterial susceptibility to host defence mechanisms (Finch and Brown, 1978; Anwar *et al*, 1983b), antibiotics (Finch and Brown, 1975; Dean *et al*, 1977; Brown *et al* 1979) and disinfectants, antiseptics and preservatives (Cozens and Brown, 1983), hence influencing the capacity of the bacteria to cause disease.

The environment of bacteria growing *in vivo* in an infection is undoubtedly different in many respects to that of organisms cultivated in the laboratory (Dalhoff, 1985), especially if the latter are grown in batch culture in conventional nutrient-rich media (Brown and Williams, 1985b). In an infection, bacteria are exposed to numerous hazards such as the onslaught of host immunological defences, changes in temperature as well as

possible nutrient limitations. Consequently, it can be predicted that such bacteria will possess significantly different characteristics from their *in vitro* counterparts. Although certain knowledge about the chemical and physical composition of the host at different sites of infection has been established, it is less easy to evaluate which of the numerous and often complex factors are important in influencing the pathogen (Brown and Williams, 1985b).

It has long been recognised that virulence may progressively be lost on subculture *in vitro* but is frequently restored by animal passage (Olitzki and Godinger, 1963; Smith, 1976 and 1977; Watson, 1982). Resistance to serum killing and phagocytosis has also been shown to increase following growth *in vivo* (Penn *et al*, 1977; Finn *et al*, 1982; Dalhoff, 1983). Many different factors may contribute to these findings, including both selection and phenotypic variation. However, the mechanisms involved can only be elucidated by investigating the bacteria *in situ* in infection. Electron microscopy has provided a valuable tool in this respect and has yielded considerable information about the ultrastructure of bacteria growing *in vivo*. This has led to the important discovery that the mode of growth adopted by bacteria in certain infections is largely in the form of adherent microcolonies on tissue surfaces (Costerton *et al*, 1979; Costerton *et al*, 1981; Costerton *et al*, 1985) or on the surface of implanted prostheses (Marrie *et al*, 1983; Marrie and Costerton, 1984: Gristina and Costerton, 1985). The cells are enmeshed in a predominantly polysaccharide matrix or glycocalyx and are not growing in a liquid medium as in most *in vitro* studies. To understand more of the general biochemistry and envelope composition of *in vivo* bacteria, however, the *in situ* cells need to be harvested directly. There are many problems associated with this. For example, it may be difficult first to obtain a pure culture of bacteria in numbers sufficient to study and second subsequently to separate the cells

from host tissue. Several researchers have attempted to overcome these problems by using chamber implants (Penn *et al*, 1977; Veale *et al*, 1977; Finn *et al*, 1982) or granuloma pouches (Dalhoff, 1982; Kroll, 1982), so localizing the infection and simplifying the procedures necessary to recover the pure bacteria. Although a significant improvement on batch culture *in vitro* in nutrient excess, the chamber implants are not ideal in that whilst soluble humoral factors and antibodies may penetrate, cellular antimicrobial defence factors are excluded. Thus, the bacteria are not subjected to the true *in vivo* environment. Neutropenic animals have been used to increase yields of bacteria and to ease purification (Zak and Kradolfer, 1979; Kadurugamuwa, 1985). However, the latter also have limitations unless the aim is to mimic infections in immunosuppressed patients. Furthermore, if *in vitro* grown cells are used as an inoculum, it should be remembered that the organisms will not exhibit *in vivo* surface characteristics for several generations (Williams *et al*, 1984). Finally, it is most frequently the free floating planktonic bacteria that are studied despite increasing evidence that the sessile population of cells attached to surfaces are perhaps more relevant in many infectious states (Costerton *et al*, 1985 and 1987). The properties of these cells may be vastly different from those of corresponding planktonic bacteria.

Nevertheless, such studies have yielded important evidence of the differing characteristics of *in vivo* and *in vitro* bacteria. Recently, a few researchers have succeeded in studying bacteria *in situ* in animal infections without the use of implants (Griffiths *et al*, 1983; Sciortino and Finkelstein, 1983). More importantly, bacteria have been recovered directly from certain human infections, so providing a valuable insight into selected structural properties of such microorganisms (Brown *et al*, 1984; Lam *et al*, 1984, Shand *et al*, 1985).

For the remaining part of this section, parameters known *in vitro* to influence bacterial properties or mode of growth will be considered individually, including their possible relevance to the *in vivo* situation. Any direct evidence that such factors exert similar effects on bacteria *in situ* in an infection will additionally be discussed. The selected parameters include nutrient availability, with a separate section on the effect of iron-restriction, growth rate, growth on a surface and sub-MIC drug concentrations.

1.1.1. Nutrient availability: *in vitro* studies.

1.1.1.1. Response to iron deprivation.

It is generally true to say that bacteria have an absolute requirement for iron, as indeed do all forms of life, although it does appear that certain strains of *Lactobacilli* have derived the means of surviving without the element (Archibald, 1983). Iron plays an essential role in a diverse range of biochemical and metabolic processes. It is a vital constituent of the cytochrome enzymes involved in electron transport (Neilands, 1974) and of ribonucleotide reductase enzymes required for DNA replication (Reichard and Ehrenberg, 1983) as well as a host of other enzymes and proteins involved in microbial metabolism, well reviewed by Neilands (1974) and Jacobs and Worwood (1974 and 1980). As a consequence, growth in an iron-depleted medium induces the bacteria to respond in a number of ways designed to maintain iron supplies to the cell. In the environment iron largely occurs in a highly insoluble form, hence many bacteria synthesize specific ligands (siderophores) to solubilize and transport the metal (Neilands, 1981). A number of siderophores have been described, the majority of which are phenolic (catechols), or hydroxamic acid compounds. The most well-characterized siderophore is the enterobactin of *Escherichia, Klebsiella* and *Salmonella* species. This molecule, a cyclic

trimer of dihydroxybenzoyl serine, is produced only under conditions of low iron availability and is the most effective iron chelating compound known (affinity constant 10^{52}; Neilands, 1981). The chelator is used only once for transporting iron into the cell, since in order to release the iron, the cyclic triester linkages of Fe^{3+}-enterobactin require cleavage by a specific esterase. Ultimately, 2,3-dihydroxybezoyl serine is produced and is subsequently discarded (Rosenberg and Young, 1984). Many strains of *E. coli* carrying the colicin V plasmid (pColV) synthesize an additional hydroxamate-type siderophore (aerobactin) encoded by the plasmid (Warner *et al*, 1981; Crosa, 1984). Recent studies have shown, in fact, that genes for aerobactin synthesis may be located on the plasmid or in the chromosome (Montgomerie *et al*, 1984). Aerobactin is considerably inferior to enterobactin in terms of its iron chelating capacity (affinity constant 10^{23}, Harris *et al*, 1979), however, it can be recycled following simple reduction of Fe^{3+} to Fe^{2+} within the cell (Braun *et al*, 1984). Therefore, it provides an energetically less expensive method of assimilating iron than enterobactin. Furthermore, in the *in vivo* environment serum antibodies for enterobactin have been demonstrated which could reduce the effectiveness of iron uptake by this siderophore (Moore *et al*, 1980), so favouring the aerobactin system. The concentration of available enterobactin in serum may also be reduced due to binding to albumin (Konopka and Neilands, 1984), whereas aerobactin is unaffected by serum proteins (Williams and Carbonetti, 1986). Certain *Enterobacteriaceae* are also capable of obtaining iron via a variety of hydroxamate-type siderophores including ferrichrome and ferrioxamine B, produced not by themselves, but by other microorganisms (Leong and Neilands, 1976; Konisky, 1979). In addition, certain strains of *E. coli* possess a citrate-mediated iron-transport system induced when the bacteria are grown in an iron-limited medium containing citrate (Frost and Rosenberg, 1973; Rosenberg and Young, 1974). *Pseudomonas* species are known

to produce several siderophores during iron restriction, ferribactin, ferrioxamine, pyoverdin, pyochelin and pseudobactin (Liu and Shokrani, 1978; Cox and Graham, 1979; Neilands, 1982; Yang and Leong, 1984; Cox and Adams, 1985). Pyoverdin and pyochelin are considered to be the most valuable siderophores during an infection and, of these, pyoverdin is the more important for growth in human serum, pyochelin synthesis being minimal (Ankenbauer *et al*, 1985). Pyochelin has a low affinity constant for iron (5 x 10^5, Cox and Graham, 1979) but that of pyoverdin is much higher (10^{32}, Wendenbaum *et al*, 1983). Recent work (Sriyosachati and Cox, 1986) demonstrated that *P. aeruginosa* placed across a dialysis membrane from [^{55}Fe] transferrin caused the movement of ^{55}Fe to the bacterial side of the membrane. This did not occur when either pyochelin or pyoverdin alone were used except when the medium was acidified from physiological pH 7.4 to between pH 5 and 6. It appeared that when bacteria were present they themselves produced acid from glucose metabolism so reducing the pH of the environment and enabling the siderophores to operate.

Growth in an iron-depleted medium also induces expression of several high molecular weight proteins (70 to 100K) in the outer membrane (OM) of a wide range of Gram-negative bacteria, thought to act as receptors for the uptake of iron-siderophore complexes (Neilands, 1982). The molecular weights of some siderophores exceed the diffusion limits of water-filled pores in the OM (Nikaido, 1979) hence the need for a specific uptake system. However, iron-citrate is small enough to diffuse through the porins but an outer membrane receptor protein is still synthesized. This suggests that such receptors are necessary to satisfy the iron requirements of the cell, perhaps by concentrating the iron-siderophore complexes at the membrane surface. The iron-regulated membrane proteins (IRMPs) of *E. coli* have been well-studied and are designated by molecular weight. An 81K protein (Fep A protein, product of fep gene) is the receptor for Fe^{3+}-

enterobactin (Hollifield and Neilands, 1978; Konisky, 1979), and a 78K protein (Fhu A protein, product of ton A gene) is the receptor for ferrichrome (Konisky, 1979; Braun and Hantke, 1981). A protein of molecular weight 85K (Fec A) is part of the citrate transport system, produced only in iron-restricted media containing citrate (Frost and Rosenberg, 1973; Hussein *et al*, 1981) and two further proteins (74K, product of cir gene and 83K) whose functions are unclear are also induced under iron-limitation. Finally, another 74K protein expressed only by strains lacking the Cir protein, is involved in aerobactin-mediated uptake of iron (Grewal *et al*, 1982). Iron limitation in *Pseudomonas* species induces a number of IRMPs (Meyer *et al*, 1979; Ohkawa *et al*, 1980) some of which, at least, are presumed to function as receptors for iron-uptake. However, this has not yet been shown directly, except for *P. syringae* pv. *syringae* in which identification of the pyoverdin receptor has recently been reported (Cody and Gross, 1987). The genes coding for these proteins have not been mapped. A low molecular weight (14K) iron-regulated protein has been reported in *P. aeruginosa* (Sokol and Woods, 1983) (see section 4.4).

Other changes are imposed on bacteria grown under iron-stress including modification of tRNA molecules, as demonstrated in *E. coli* (Griffiths and Humphreys, 1978). Evidence suggests that the latter have lowered translational efficiencies and relieve transcriptional termination at the attenuators of certain operons of the aromatic amino acid biosynthetic pathway, so promoting expression of these operons (Buck and Griffiths, 1982). A branch of this pathway is involved in enterobactin synthesis, and multiple control of the operons may provide the cell with regulatory flexibility during growth in a low iron environment (Griffiths, 1983). Reduced activity of bacterial ribosomes is also reported to occur under iron-deprivation (Iorio and Plocke, 1981), as well as changes in the penicillin binding protein profiles as seen in *P. aeruginosa*, a phenomenon

additionally linked with growth rate (Turnowsky *et al*, 1983). Finally, toxin (Bjorn *et al*, 1978 and 1979; Sokol *et al*, 1982) and extracellular enzyme production (Wiersma *et al*, 1978; Ombaka *et al*, 1983) may be influenced by iron levels.

1.1.1.2. Nutrients other than iron.

There are a vast number of reports in the literature concerning the effects of nutrient-depletion on all aspects of bacterial cell physiology and structure, a subject thoroughly reviewed by Brown and Williams (1985b). Induction or repression of a variety of outer membrane proteins (OMPs) is a common response of bacteria in such conditions, some of which may be involved in uptake mechanisms for the nutrient required. Under phosphate limitation, *E. coli* expresses an additional 36K porin protein, Phoe E, possessing channels selective for charged molecules including phosphorylated compounds (Lugtenberg and van Alphen, 1983). *P. aeruginosa* produces a similar phosphate regulated porin (protein P) thought to have a role in phosphate transport into the cell (Hancock *et al*, 1982). Sulphate limitation induces 2 OMPs (15 and 19K) in *E. coli* K12 of unspecified function (Lugtenberg and van Alphen, 1983), whilst magnesium deficiency results in enhanced expression of protein H_1 in *P. aeruginosa* (Nicas and Hancock, 1980) with a concomitant increase in resistance to gentamicin, polymyxin and EDTA. Magnesium depletion also results in a much simplified OMP profile for *P. cepacia* compared to iron-limited and nutrient broth grown cells (Anwar *et al*, 1983a). The same organism grown in continuous culture expresses a 46K OMP under carbon-limitation but not under magnesium-limitation (Taylor, 1984). Other specific proteins were observed in the OMs of *K. pneumoniae* grown in continuous culture under potassium, carbon, sulphate and phosphate limitations (Sterkenberg *et al*, 1984).

Nutrient depletion is also known to affect other components of the cell envelope, with changes in phospholipids (Cozens and Brown, 1981) particularly in magnesium-depleted cells (Günther *et al*, 1975), fatty acids (Minnikin *et al*, 1971, a and b) and cation content (Kenward *et al*, 1979). Additionally, marked changes in LPS composition have been noted (Tempest and Ellwood, 1969; Dean *et al*, 1977, Gilbert and Brown, 1978; Taylor, 1984) including variation in the core region heptose content under magnesium-limitation (Day and Marceau-Day, 1982). Exopolysaccharide production is also influenced by nutrient availability (Ombaka *et al*, 1983; Chan *et al*, 1984)

In some instances, phenotypic modification of the bacterial cell envelope in response to nutrient availability has been correlated with changes in the sensitivity of the microorganism to antimicrobial agents (Finch and Brown, 1975; Dorrer and Teuber, 1977; Brown and Melling, 1969) and serum killing (Anwar *et al*, 1983b; Taylor, 1984). This may have important consequences in terms of pathogenicity.

1.1.2. Nutrient availability *in vivo*

1.1.2.1. Availability of iron.

Although knowledge of nutrient limitations in the *in vivo* environment is not extensive, there is considerable information concerning the restriction of iron in infection

It has been clearly demonstrated that both *E. coli* recovered directly from the peritoneum of lethally infected guinea pigs (Griffiths *et al*, 1983) and 4 strains of *Vibrio cholerae* infecting the intact intestinal tracts of infant rabbits (Sciortino and Finkelstein, 1983) expressed high molecular weight IRMPs, providing strong evidence that these bacteria were growing under iron-depleted conditions *in vivo*. Moreover, the siderophore enterobactin and its degradation products were detected in the peritoneal

washings from the *E. coli* infected guinea pigs, further implicating the iron-restricted nature of the infection site. Iron-limited growth in human infection was first reported by Brown *et al* (1984) who observed IRMPs in the OMs of *P. aeruginosa* harvested without subculture from the sputum of a patient with cystic fibrosis (CF). Subsequently, Lam *et al* (1984) recovered strains of *E. coli*, *P. mirabilis* and *K. pneumoniae* from the urines of patients with urinary tract infection and found that 5 of 12 strains exhibited 2 or more IRMPs. Interestingly, fewer porin proteins were observed in these *in vivo* cells compared to the same isolates cultivated in TSB. IRMPs were also present in the OMs of *K. pneumoniae* and *P. mirabilis* isolated without subculture from infected human urine by Shand *et al* (1985). However, only 3 IRMPs were expressed by *K. pneumoniae* direct from the urine, whereas 6 or more such proteins were induced in the same isolate grown in a chemically-defined media lacking iron, or in other *Klebsiella* strains grown in serum or iron-deficient media (Williams *et al*, 1984). This implies that factors other than solely iron-deprivation may influence the regulation of these proteins. The altered tRNA species noted in bacteria *in vitro* under iron stress were also observed in pathogenic *E. coli* recovered directly from infected animals (Griffiths *et al*, 1978). Indirect evidence of high affinity iron uptake systems induced in bacteria growing *in vivo* includes demonstration of antibodies to enterobactin in normal human serum (Moore *et al*, 1980; Moore and Earhart, 1981) and to the ferric enterobactin receptor (Griffiths *et al*, 1985). Additional antibodies to *P. aeruginosa* IRMPs were detected in serum from a CF patient with *Pseudomonas* lung infection (Anwar *et al*, 1984) and, similarly, serum from patients with UTI were found to contain antibodies to the IRMPs of *K. pneumoniae* and *P. mirabilis* (Shand *et al*, 1985).

It is now clearly recognized that although there is plenty of iron present in the body fluids of humans and animals, the amount of free iron

available to bacteria is extremely small (Griffiths, 1987). The majority of iron is located intracellularly as ferritin, haemosiderin, haemoglobin and myoglobin and that which exists in body fluids is tightly bound to carrier proteins, transferrin in serum and lymph and lactoferrin in external secretions and polymorphonuclear leucocytes (PMNs) (Bullen and Armstrong, 1979). The latter have approximate association constants of 10^{36} for iron (Bullen, 1981; Griffiths, 1983) but in humans are only partly saturated (30 to 40%), an important safeguard in case of sudden influx of the metal into plasma. Although, all forms of life require solubilized iron the free form of the element is not desirable since it may react and produce toxic radicals (Griffiths, 1987). As a consequence, the amount of iron available to infecting bacteria is in the order of 10^{-18}M, way below the levels required for proliferation (Bullen *et al*, 1978). The biological chelators or transferrins (transferrin, lactoferrin) are glycoproteins of molecular weight in the range 75 to 80K, with the capacity of reversibly binding 2 ferric irons (Bullen *et al*, 1978). The first transferrin was discovered and purified from human plasma in 1946 by Schade and Caroline, who also demonstrated that it inhibited microbial growth. Lactoferrin, which differs from transferrin in that it has a higher affinity for iron and will bind at a lower pH (Aisen and Liebman, 1972) is one of the components of the specific granules of PMNs. Not itself bactericidal, lactoferrin may maintain a low iron environment within the acidic phagolysosome such that other components can destroy ingested bacteria (Bullen and Armstrong, 1979). It is also required to generate oxidising hydroxyl radicals within the PMNs (Ambruso and Johnston, 1981).

It is understood that during infection the host reduces the total amount of iron bound to serum transferrin (Cartwright *et al*, 1946). A similar hypoferraemia can be induced experimentally by injecting endotoxin which causes the release of a compound from PMNs (Baker and Wilson, 1965).

The latter, now termed interleukin 1 (Dinarello, 1984), is known to stimulate fever, leucocytosis and release of the contents of polymorph granules (Leffel and Spitznagel, 1974). It has been proposed that hypoferraemia is then achieved by the lactoferrin released from polymorphs which can remove iron from transferrin. Fe^{3+}-lactoferrin complexes are subsequently thought to be taken up by macrophages and removed from the circulation by the reticuloendothelial system (van Snick *et al*, 1974). However, this theory has been challenged by Baynes *et al* (1986). Another hypothesis is that as part of the inflammation response, synthesis of ferritin, an iron storage protein, increases and iron is deviated into the ferritin and not, therefore, released into the plasma (Konijn and Hershko, 1977).

The bacterial siderophores described (section 1.1.1.1.) all have the capacity to remove iron from host proteins, so enabling bacteria to acquire the iron they need. In addition, under certain circumstances pathogenic bacteria can obtain iron *in vivo* from cell free haem or haemoglobin although the mechanisms involved are, as yet, unknown (Griffiths, 1987). Bacteria lacking high affinity uptake systems are rarely pathogenic and host restriction of iron is an important non-specific host defence mechanism. Indeed, there is considerable evidence that an iron rich environment in the host produced experimentally as summarized by Griffiths (1983), or by trauma, or in certain disease states can exacerbate infections (Bullen, 1981; Griffiths, 1983; Finkelstein *et al*, 1983). The excess iron may not only promote bacterial growth, but also may impair the function of monocytes and granulocytes (van Asbeck *et al*, 1984). Lowered transferrin levels in certain host conditions such as malnutrition and graft versus host disease (Hunter *et al*, 1984), or increased saturation of transferrin as seen in patients following chemotherapy (Gordeuk *et al*, 1986) may also predispose to infection, hence emphasizing the importance of this element.

1.1.2.2. Nutrients other than iron.

As yet there is little information concerning the availability of nutrients apart from iron during infection (Brown, 1977). However, *P. aeruginosa* recovered directly from the CF infected lung strongly expressed protein H_1 (Brown *et al*, 1984) which can be induced by magnesium deprivation (Nicas and Hancock, 1980). Hence, this may suggest that the lung environment is deficient in magnesium which, in addition, may influence susceptibility of the bacteria to aminoglycoside drugs. Broughton *et al* (1968) reported on the fall in serum magnesium in a proportion of patients with burns and, although levels of magnesium in the burn tissue itself were not established, it is likely that saline baths frequently employed in burn wounds, may further reduce the concentration of this metal ion, in turn affecting bacterial properties.

Levels of other trace metals including zinc and copper are known to fluctuate during fever and inflammation (Pekarek and Engelhardt, 1981) and it is possible that unsaturated transferrin molecules may be able to chelate cations in addition to iron, even if affinity constants for these cations are lower (Brown and Williams, 1985b). A growth limiting effect has been suggested for inorganic phosphate (Weinberg, 1974).

There is data to indicate that cells grown *in vivo* are metabolically more active than their *in vitro* counterparts (Segal and Bloch, 1956). This fact may reflect reduced nutrient supply in the infected foci so resulting in increased biosynthesis of the required nutrients or, of enzymes or molecules to acquire the nutrients from host proteins (Dalhoff, 1985). Finally, it was shown in a granuloma pouch model that *Proteus* and *Pseudomonas* species produced β-lactamase even in untreated animals, but not *in vitro*, which the researchers postulate may be due to the presence of aromatic amino acids in body fluids known to be potent β-lactamase inducers (Dalhoff and Cullman, 1984).

There are many more questions to be answered in terms of nutrient supply and composition of the growth environment *in vivo*, but undoubtedly growth of bacteria in conventional laboratory media bears little resemblance to the conditions in infection.

1.1.3. Growth Rate: *in vitro* studies

Chemostat culturing offers many advantages over batch culturing, enabling studies of cells grown under different growth rates to be performed. The growth rate itself is determined by the dilution rate of a culture in which one nutrient is limiting (Tempest, 1970).

There are numerous reports in the literature concerning the effect of growth rate on many envelope properties. The alterations observed at different growth rates are often dependent on the limiting nutrient. Examples include the reduced LPS content of slow growing *Neisseria gonorrhoeae* in a carbon-limited chemostat with corresponding increased hydrophobicity and sensitivity to serum killing (Morse *et al*, 1983). Conversely, the LPS content of *P. aeruginosa* decreased with increasing growth rate in both carbon and magnesium limited cultures, the level of LPS appearing to determine the degree of penetration of the cell envelope by chlorinated phenols (Gilbert and Brown, 1978). In addition, fast growing cells contained less phospholipids and more fatty acids than slow growing cells. A recent paper has described more specific alterations in the LPS of *E. coli* grown in continuous culture at different growth rates. It was found that under both carbon and magnesium limitation much less high molecular weight LPS was produced at high dilution rates and, that the small amount of 'O'-polysaccharide present lacked amino sugars and thus differed chemically from that produced at low growth rates (Dodds *et al*, 1987). The predominant form of LPS in the former cells was S-R LPS and the authors suggest that the altered growth rates affect the activity of

enzymes (ligase and polymerase) involved in LPS biosynthesis. A smooth to rough transition was also noted for the LPS of *S. enteritidis* at high growth rates (Collins (1964). A 39K peptidoglycan-associated protein of *K. pneumoniae* expressed under carbon limitation at fast growth rates was found to be repressed when the growth rate was reduced (Lodge *et al*, 1986). Furthermore, these researchers found that lowering the iron content of the carbon limited chemostats resulted in induction of IRMPs only at the fast growth rate. *P. aeruginosa* grown in continuous culture under magnesium limitation at a slow growth rate was more resistant to phagocytosis and associated cationic proteins than when growth rate was increased (Finch and Brown, 1978) and the same organism became more sensitive to EDTA or gentamicin at higher growth rates under carbon limitation (Dean *et al*, 1977). Finally, exopolysaccharide production by *P. aeruginosa* was shown to be influenced by the rate of growth (Mian *et al*, 1978).

It is obvious, therefore, that varying the growth rate of cells *in vitro* can have a complex array of effects on bacterial envelope properties, emphasizing once more the plasticity of the cell envelope.

1.1.4. Growth rate: *in vivo* studies.

Many researchers have attempted to define growth rates of bacteria in *in vivo* infections. Meynell (1959) developed techniques which allowed independent measurement of the rate of bacterial division and death rate after challenge of animals with strains of *E. coli* or *S. typhimurium*. A known proportion of the cells carried a non-replicating genetic marker such as a superinfecting phage or an abortively transduced phage which is distributed to only 1 of the 2 daughter cells. At the end of a specified time the proportion of cells containing the marker enabled determination of the number of generations which had occurred over the time period. The doubling time of *S. typhimurium* was reported as being 5 to 10 h in the

spleen (Maw and Meynell, 1968) and 6 h in the alimentary tract of mice (Meynell and Subbaiah, 1963). This approach relies on the assumptions that cells divide equally with or without the phage, and that they have an equal survival probability. However, it is known that sensitivity to host defences and antibiotics may vary considerably depending on presence or absence of phage (Eudy and Burrous, 1973).

Radiolabelled bacteria have also been used to measure *in vivo* replication. Eudy and Burrous (1973) determined generation times of *P. mirabilis* and *E. coli* in experimental kidney infections using radioisotope techniques. Their results suggested a more rapid growth rate during the first 7 h following infection with doubling times of 2.3 and 0.9 h respectively, followed by a considerable increase to approximately 20 h in the subsequent 41-h period. Similar techniques were used by Baselski *et al* (1978) who reported multiplication of *V. cholerae* in the upper bowels of mice at a rate of 1.5 generations in 4 h, and Sigel *et al* (1981) who estimated doubling times of approximately 1 h for *V. cholerae* strains in a mouse model.

Such results have suggested that growth rates *in vivo* are, on the whole, slower and in some cases considerably slower than *in vitro*, and it was considered that bacteriostatic factors elaborated by the host or substrate limitations were in part responsible (Smith, 1980). Nonetheless, certain reports have recently indicated that replication rates may be higher than previously thought, perhaps depending on the site of infection and the bacterial strains used in the study. Rubin (1986) calculated growth rates of *H. influenzae* type b inoculated IV into rats and determined mean generation times of 34+/- 5 min in asplenic rats and 82+/-39 min in sham operated rats compared to 26+/-1 min in broth, implying that impairment of host clearance mechanisms by splenectomy accounted for the significantly shorter IV generation times in the asplenic rats. These results, however,

were net rates after host clearance of microorganisms. Morris Hooke *et al* (1985) evaluated mean generation times during the first stages of infection using temperature sensitive mutants which cease replication at 36°C but remain viable under these conditions for 18 h. These bacteria were inoculated in combination with wild type parents and changes in the ratios of the 2 organisms evaluated. Values of 33 min (*E. coli*) and 20 min (*P. aeruginosa*) were calculated in the peritoneal cavities of mice, with operating host clearance machanism. In the lungs of mice a higher mean generation time for *P. aeruginosa* (56 min) was obtained, possibly due to reduced availability of nutrients and oxygen. In a subsequent paper (Sordelli *et al*, 1985) the authors comment that doubling times do vary according to conditions such as inoculating dose and the temperature at which the animals are kept. Furthermore, it should be noted that the media used to cultivate the initial inocula may well affect the growth rate values obtained, particularly in the first few hours after injection.

Despite the variations in reported growth rates, partly due to use of different techniques, bacterial strains and sites of infection, it is well recognized that mean generation times *in vivo* may differ markedly from cells cultivated *in vitro*, in turn influencing the structure and composition of the cell envelope. Clearly, the growth rate of invading bacteria is paramount in determining the outcome of an infection, the shorter the doubling time the sooner the attainment of a bacterial population sufficient to harm the host.

1.1.5. Adherence and Surface growth: *in vitro* studies.

It has become increasingly obvious in the last 20 years that bacteria in a wide variety of environments show a natural tendency to adhere to available surfaces and, soon after attachment, to produce large amounts of exopolysaccharide which envelopes the cells and in which they divide and

form microcolonies (Costerton and Marrie, 1983). Clear evidence of this mode of growth did not initially come from *in vitro* studies since the nutrient-rich liquid media routinely used to cultivate bacteria in the laboratory favours overgrowth of mobile cells as opposed to a sessile population (Costerton *et al*, 1981). However, away from this artificial environment in natural aquatic habitats or in infection, bacteria encounter numerous adverse conditions and, as a consequence, the majority of cells adopt the more defensive adherent mode of growth, enveloped in the glycocalyx (Costerton and Marrie, 1983). Electron microscopy (EM) studies first brought to light existence of the glycocalyx, although early EM preparations did not effectively visualize the stucture. Even with the advent of polyanion-specific stains such as ruthenium red (Luft, 1971) the glycocalyces were only seen as condensed, electron dense aggregates on the bacterial cell surface due to the dehydration steps required for EM (Costerton *et al*, 1985). The polysaccharide matrices are highly hydrated in their natural state consisting of 99% water (Sutherland, 1977) and dehydration causes their almost total collapse. However, the finely fibrillar glycocalyx was eventually observed in its true state following development of techniques to stabilize the structure using lectins (Birdsell *et al*, 1975) and specific antibodies (Mackie *et al*, 1979; Chan *et al*, 1982).

Surface-growing microcolonies were initially demonstrated in studies of industrial aquatic systems where it was found that for various Gram-negative microorganisms, the numbers of biofilm-encased bacteria exceeded free-floating forms by approximately 1000 to 10,000 times (Geesey *et al*, 1978). Such bacteria were attached to submerged surfaces including clay particles and rocks. Moreover, autoradiographic techniques (Fletcher, 1979) and heterotrophic potential studies of aquatic bacteria growing in thick biofilms (Ladd *et al*, 1979) indicate that more than 70% of the cells are metabolically active. The chemical nature of the glycocalyx may be complex

and is often variable depending on growth conditions. The glycocalyx itself, as defined by Costerton *et al* (1981) represents any polysaccharide-containing structure of bacterial origin lying outside the integral elements of the outer membrane of Gram-negative cells and the peptidoglycan of Gram-positive cells. As such, this includes both the S layers of glycoprotein subunits described by Sleytr (1978), or capsules which may be either rigid, flexible, integral or peripheral (Costerton *et al*, 1981). Bacterial capsules have been well reviewed in terms of their chemical composition by Sutherland (1977) and may either be comprised of simple homopolymers or very complex heteropolymers of a wide variety of monosaccharides including neutral hexoses, 6-deoxyhexoses, polyols, uronic acids and amino sugars. These frequently may be substituted with phosphate, formate, pyruvate and succinate groups. Although certain of the literature presents a confusing and conflicting picture of the composition of exopolysaccharides from various bacteria, this was in part due to analysis of material from cultures in which some cells had lysed resulting in contamination with intracellular components (Costerton *et al*, 1981). An additional factor is that synthesis of exopolysaccharides is dependent on physiological conditions including concentration of certain cations, and the ratio of carbon to nitrogen , as well as growth on solids as opposed to in a liquid media (Sutherland, 1977; Chan *et al*, 1984). Divalent cations have been shown to play a role in linking S layer glycoproteins to the cell wall (Chester and Murray, 1978) a mechanism which may also operate in attaching the glycocalyx. Interactions between LPS polysaccharides and glycocalyx may additionally be important in this respect.

Hence, it becomes apparent that the surface structures of adherent cells are markedly different from those of their planktonic equivalents. The former possess a fibrous polysaccharide layer, the latter lack this structure and thus expose other components of the cell envelope including

LPS and OMPs. Properties such as adhesion, antigenicity, hydrophobicity and antibiotic sensitivity, therefore, may show wide variation between the 2 cell types.

It has since been recognized that inclusion of a surfactant (Govan, 1975) or low levels of the antibiotic carbenicillin (Govan and Fyfe, 1978) in the liquid culture medium *in vitro* encourages glycocalyx formation and vast microcolonies are visible both settling to the bottom of the flask and adherent to the vessel walls at the medium-air interface (Lam *et al*, 1980). Moreover, a device called the Robbins Biofilm Sampler (McCoy and Costerton, 1982) has been developed to enable formation of adherent sessile bacterial biofilms *in vitro* on a variety of surfaces. The surfaces are mounted on 'studs' which can be aseptically removed from the device and the biofilms studied directly. Using this device it has been possible to demonstrate *in vitro* that cells enclosed within biofilms are significantly more resistant to the action of chemical biocides used in the industrial environment (Ruseska *et al*, 1982). Presumably they are protected, in part, by the anionic matrix which must be saturated with the biocide before penetration into the bacterial cells can occur. The original Robbins device has subsequently been modified to incorporate inert plastic surfaces such as those used in a range of medical prostheses. The device was then immersed in an artificial urine milieu inoculated with *P. aeruginosa*, and used to show that the biofilm bacteria which developed on the plastic surfaces were extremely resistant to antibiotics (Nickel *et al*, 1985b). Exposure to 1000mg/l tobramycin for 12 h failed to kill all the sessile bacteria, whereas planktonic cells of the same strain were killed by 50mg/l. Alternative studies have indicated that this mode of growth also provides bacteria with a measure of protection from surfactants (Govan, 1975), antibodies (Baltimore and Mitchell, 1980) and phagocytic cells (Schwarzmann and Boring, 1971).

These observations all have important implications with regard to *in vivo* infections, stressing once again the dangers of extrapolating too much information from cultures of bacteria grown in conventional liquid media.

1.1.6. Adherence and surface growth *in vivo*.

There are numerous infection types in which the biofilm mode of growth has been directly demonstrated by EM techniques and may well be responsible for the persistent nature of such infections. Examples include bacterial endocarditis in which pathogens adhere to tissues of the endocardium in large microcolonies of macroscopic proportions termed 'vegetations'. These bacteria are inherently resistant to antibiotic therapy. *Streptococci* species are frequently implicated in this disease and TEM studies have visualized extensive dextran glycocalyces surrounding cells in the vegetations, both in human infection and in experimental endocarditis in rabbits (Mills *et al*, 1984). The capacity of this group of bacteria to produce large amounts of extracellular dextran is thought to correlate with their ability to form such resistant vegetations (Costerton and Marrie, 1983).

Osteomyelitis is a particularly chronic bacterial disease and the direct examination of infected bone has shown the presence of glycocalyx-enclosed microcolonies of numerous pathogens causing this infection (Costerton *et al*, 1985). In an animal model of osteomyelitis rabbits with fully intact humoral and cellular defence mechanisms could not eradicate the causative pathogens which were seen to exist in microcolonies (Mayberry-Carson *et al*, 1984).

A similar condition is apparent in the lungs of CF patients suffering from chronic *P. aeruginosa* infection, and of rats in a model of chronic lung infection (Lam *et al*, 1980). The bacteria commonly produce large

amounts of a polymer composed, in part, of alginate material. CF patients are frequently hosts to the infecting bacteria for upwards of 20 years without resolution. Neither is there evidence of general toxaemia or bacteraemia in these patients, partly due to the mounting of a strong immune response to the bacteria over the years of exposure. Such host defences can effectively destroy any potentially invasive planktonic cells released from the lung environment (Costerton *et al*, 1979) but fail to eradicate the bacteria embedded in their extensive polysaccharide glycocalyx.

P. aeruginosa does not exist in adherent biofilms in all infections, however, and it appears that the mode of growth selected by the bacteria largely depends on the immunological status of the host. In burn patients whose defences against infection are severely compromised both in terms of destruction of the physical barrier to invading microbes and, as a result of post-burn immunosuppression (Pruitt *et al*, 1983; Munster, 1984), mobile bacteria can rapidly proliferate, producing toxins and enzymes to lyse the eschar. The consequences may be invasive wound infection, bacteraemia and death. These bacteria are not forced into the sessile mode by operative defence mechanisms (Costerton *et al*, 1985) and an acute, life-threatening infection can develop. If host immunity is essentially intact, the more defensive form of growth is thought to be necessary for survival.

The advent of modern invasive medical techniques involving implantation into patients of plastic and metal prostheses, has brought with it an increasing number of infections associated with the implants. The biologically inert materials provide an ideal surface for colonization by bacteria, and EM studies have shown, once again, that the bacteria preferentially grow in coherent biofilms enmeshed in a glycocalyx envelope (Marrie *et al*, 1983; Marrie and Costerton, 1984; Gristina and Costerton, 1985). The structure of these prosthesis biofilms is very similar to those

observed in natural ecosystems and colonizing tissue surfaces. Examples of this include the biofilms visualized on the surfaces of intrauterine contraceptive devices (Marrie and Costerton, 1983a) in which many different morphological types of bacteria were seen as well as microcolonies containing a single bacterial species. Examination of intravenous and intraarterial catheters recovered directly from patients revealed confluent biofilms on more than half the prostheses, largely involving *Staphylococcus epidermidis* (Marrie and Costerton, 1984). Both luminal and outer surfaces of Tenckhoff catheters (Tenckhoff and Schechter, 1968) used in continuous ambulatory peritoneal dialysis, were heavily colonized with biofilm-encased microorganisms if they had been in place for more than 1 month. Such reservoirs of bacteria are almost impossible to eliminate with antibiotics and have been implicated as a cause of recurrent peritonitis frequently seen in these patients (Marrie *et al*, 1983; Dasgupta *et al*, 1986). Infections associated with a variety of orthopaedic prostheses including sutures, joint replacements, and cardiac valves all involved bacteria growing as described (Gristina and Costerton, 1985). Finally, colonized urinary catheters showed the presence of thick biofilms (Nickel *et al*, 1985a).

Frequently, this mode of growth not only protects the bacteria from host phagocytic cells and antibiotics, but also limits their pathogenic impact on the host. Certain patients can harbour substantial populations of of bacteria on implanted catheters without experiencing any significant symptoms (Costerton *et al*, 1983). However, if host defences weaken in any way these biofilms provide a nidus of bacterial cells which may release planktonic forms and cause a more widespread debilitating infection. Infection is often only resolved by removal of the implanted devices (Costerton *et al*, 1985) and, as such, poses the most important limitation to the use of prostheses in human medicine.

1.1.7. Sub MICs of antibiotics: *in vitro* studies.

Many researchers have investigated the effect of sub-MIC concentrations of antibiotics on bacterial cell properties including susceptibility to serum killing and phagocytosis. It is apparent that at these concentrations, drugs act in a manner different from MIC or MBC concentrations, not merely causing reduced effects of the latter (Brown and Williams, 1985b). Alterations in morphology are frequently encountered. For example, filament formation is seen in many Gram-negative bacteria at sub MIC concentrations of β-lactam antibiotics (Lorian and Atkinson, 1984) and multiple cells of *Staphylococci* occurred with thick cross walls following exposure to sub-MIC's of penicillins (Lorian *et al*, 1982). Certain bacteria are rendered less resistant to serum killing (Taylor *et al*, 1982) and, more specifically to the action of complement (Storm *et al*, 1977; Vaara and Vaara, 1983) after sub MIC treatment with some drugs, albeit not all (Lorian and Atkinson, 1979). Nonetheless, sub MIC's of many antibiotics result in increased phagocytosis of both Gram-negative and Gram-positive bacteria (Gemmell, 1984; Lorian and Atkinson, 1984). Adhesive properties of many bacteria may also be affected (Tylewska *et al*, 1981; Vosbeck and Mett, 1983). Although a large number of studies have been conducted there is, as yet, little information regarding specific changes in macromolecular surface characteristics of bacteria responsible for the observed effects. In some instances, however, structural variation has been noted including for *Bacteroides fragilis* where increased phagocytosis of cells following treatment with sub MIC's of clindamycin was correlated with loss of capsule (Gemmell *et al*, 1983) and, increased serum sensitivity of *E. coli* after growth in mecillinam (sub MIC) was suggested to be due to reduced envelope polysaccharide content (Taylor *et al*, 1982).

It should be noted, however, that in these studies cells were cultivated in conventional laboratory media in batch culture which may well

have affected their response to drug treatment. In an attempt to provide growth conditions more closely resembling those *in vivo* Kadurugamuwa *et al* (1985a) examined the effect of sub MICs of cephalosporins on properties of *K. pneumoniae* grown under iron-depleted conditions. The drug concentrations used had little or no effect on growth rate. Enterochelin production and capsule formation were both markedly reduced and surface hydrophobicity increased after this treatment. Subsequent studies demonstrated directly that the loss of capsule exposed further protein antigens including the IRMPs on the surface, so that they became readily accessible to antibodies (Kadurugamuwa *et al*, 1985b). Such effects may influence the susceptibility of the cells to opsonization and phagocytosis.

1.1.8. Sub MIC's *in vivo*.

Although there is little direct knowledge of sub MIC effects *in vivo* it is recognized that during an infection treated with antibiotics many bacteria will be exposed to drug levels below their effective MIC or MBC, due to difficulties in achieving desired drug concentrations. It is also clear that at sub MIC's many drugs exert effects which although do not initially kill the bacteria, may render them more susceptible to host defence mechanisms. Certainly, altered morphological types have been noted in the blood and tissue fluids of patients receiving antimicrobial chemotherapy, but it is realized that such transformations may also have been induced by serum components and pH changes (Atkinson and Amaral, 1982).

The factors described in preceeding sections may account for certain of the unique features possessed by microorganisms growing *in vivo*. However, many of the observed characteristics of such bacteria have yet to be explained. Both *E. coli* recovered directly from the peritoneum of

lethally infected guinea pigs (Griffiths *et al*, 1983) and *P. mirabilis* and *K. pneumoniae* strains harvested from infected human urines (Sciortino and Finkelstein, 1983) showed differences other than those related to iron, in expression of OMPs. The former possessed additional proteins in their OM, the latter had fewer porins when compared to *in vitro* grown cells. Dalhoff (1983) also reported reduced synthesis of an *E. coli* porin following *in vivo* growth in a granuloma pouch and, Kroll (1982) demonstrated marked loss of protein from the OM of *K. pneumoniae* grown in a similar fashion. These and numerous other effects, a more detailed account of which has been made by Dalhoff (1985), emphasizes the current lack of knowledge in this area. Nonetheless, all the information accumulated thus far has unequivocally established that cells grown by conventional methods in the laboratory frequently bear little or no resemblance to the same organisms in infection. If improved strategies for controlling and eliminating such infections are to be sought then studies of bacteria obtained directly from sites of infection or cultivated *in vitro* under conditions more closely mimicking the *in vivo* environment are essential.

1.2. The cell envelope of *P. aeruginosa*.

The cell envelope is a complex structure that confers shape and rigidity on the cell and which regulates the organisms interaction with the environment. Its composition is extremely variable as outlined in section 1.1 and, as such, it is meaningless to describe specific components in terms of their structure and content without stating the conditions under which the organism was grown (Brown, 1975; Brown and Williams, 1985, a and b). The cell envelope of *P. aeruginosa* undoubtedly plays a major role in the pathogenesis and intrinsic resistance of this organism and hence its prominence in so wide a variety of infections in the compromised host (Costerton *et al*, 1979).

Essentially, the envelope can be divided into the inner cytoplasmic membrane (CM), the peptidoglycan layer (PG), the periplasm, the outer membrane (OM), the extracellular polysaccharide layer and surface appendages.

The CM is a bilayer composed of phospholipids and embedded proteins, both its inner and outer faces being hydrophobic due to partly exposed fatty acid moieties. CM proteins include enzymes of the electron transport chain, and proteins involved in the active transport of solutes and excretion of waste products, as well as synthesis and translocation of PG and OM components (Inouye, 1979).

The PG encloses the CM and forms the rigid backbone of the cell envelope so enabling it to withstand osmotic pressure. PG itself is a macromolecule consisting of N-acetylmuramic acid and N-acetylglucosamine molecules cross-linked by short peptide chains to form a rigid polymer. Reviews by Mirelman (1979) and Hammond *et al* (1984) provide more detailed information on PG structure and biosynthesis.

It has been proposed that the periplasm forms a gel in the space between the CM and OM, composed of hydrated PG, more highly cross-linked than the inner PG layer described (Hobot *et al*, 1984). Within the gel are oligosaccharides involved in osmolarity regulation (Lugtenberg and van Alphen, 1983) and 3 classes of low molecular weight proteins, those with a catabolic function, those which bind nutrients and those which act as enzymes involved in degradation of harmful substances including the PBPs (Hammond *et al*, 1984).

The OM and extracellular polysaccharide layer surround the periplasm and, along with surfaces appendages such as flagella and pili, are the structures which most directly interact with the environment. Hence, their composition has relevance in terms of pathogenicity and will be considered in more detail.

1.2.1. The outer membrane.

The OM is basically comprised of a bilayer containing lipopolysaccharide largely present in the outer leaflet and phospholipids mainly confined to the inner leaflet. Proteins are embedded to a greater or lesser degree in the membrane structure.

1.2.1.1. Phospholipids (PL).

The phospholipid content of the OM is qualitatively similiar to that of the CM but the proportions of the individual PLs differ. PLs have the basic structure shown in Fig. 1.1 consisting of glycerol, esterified with fatty acids on 2 carbon atoms and substituted phosphoric acid on the third. The fatty acid substituents present in *P. aeruginosa* PLs consist mainly of straight chain saturated acids (C 12:0 and C16:0) and unsaturated acids (C 16:1 and C 18:1), hydroxy acids (3-OH 10:0; 2-OH 12:0 and 3-OH 12:0) and cyclopropane acids ($17:0_{cyc}$ and $19:0_{cyc}$), the latter being characteristic of

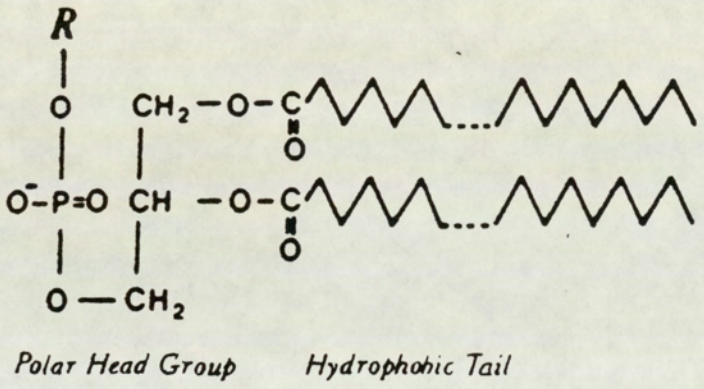


Fig. 1.1. Basic structure of bacterial phospholipids. (From Hammond *et al*, 1984).

P. aeruginosa and 2 other fluorescent pigmented species (Ikemoto *et al*, 1978).

The fatty acid composition and distribution varies with nutrient depletion (Cozens and Brown, 1981) and notably with temperature (Kropinski *et al*, 1987). The OM must contain some fluid and some non-fluid fatty acids at all temperatures leading to a membrane with a mixture of saturated and unsaturated acids. At lower temperatures the minimum amount of saturated fatty acids required is less than that needed at higher temperatures and mechanisms operate to adjust the composition of the membrane to produce a structure with the optimum fluidity for membrane function. Inclusion of cyclopropane fatty acids serves the same purpose as unsaturated fatty acids in this respect (Hammond *et al*, 1984).

1.2.1.2. Outer membrane proteins (OMPs).

Knowledge of the proteins present in the OM of *P. aeruginosa* was not obtained for a considerable time after the extensive studies of enteric bacterial OMPs, largely as a result of difficulties in separating outer and cytoplasmic membranes in this organism. More detailed information was obtained from the studies of Hancock and Nikaido (1978), Matsushita *et al* (1978) and Mizuno and Kageyama (1978) who identified a number of proteins. Initially, different nomenclature systems were applied, but the system now generally adopted is based on that described by Mizuno and Kageyama (1978, 1979, a and b) which has been subject to certain modifications (Hancock and Carey, 1979). It is well established that many of the proteins are only expressed under specific growth conditions, frequently in response to the availability of particular nutrients. The proteins have been characterized mainly according to their migration pattern following SDS-PAGE (see Fig 1.2), and 5 are described as heat modifiable since their electrophoretic mobility differs depending on the temperature of denaturation (Hancock and Carey, 1979). Proteins D_1, D_2, F, G and H_1 all migrate a shorter distance

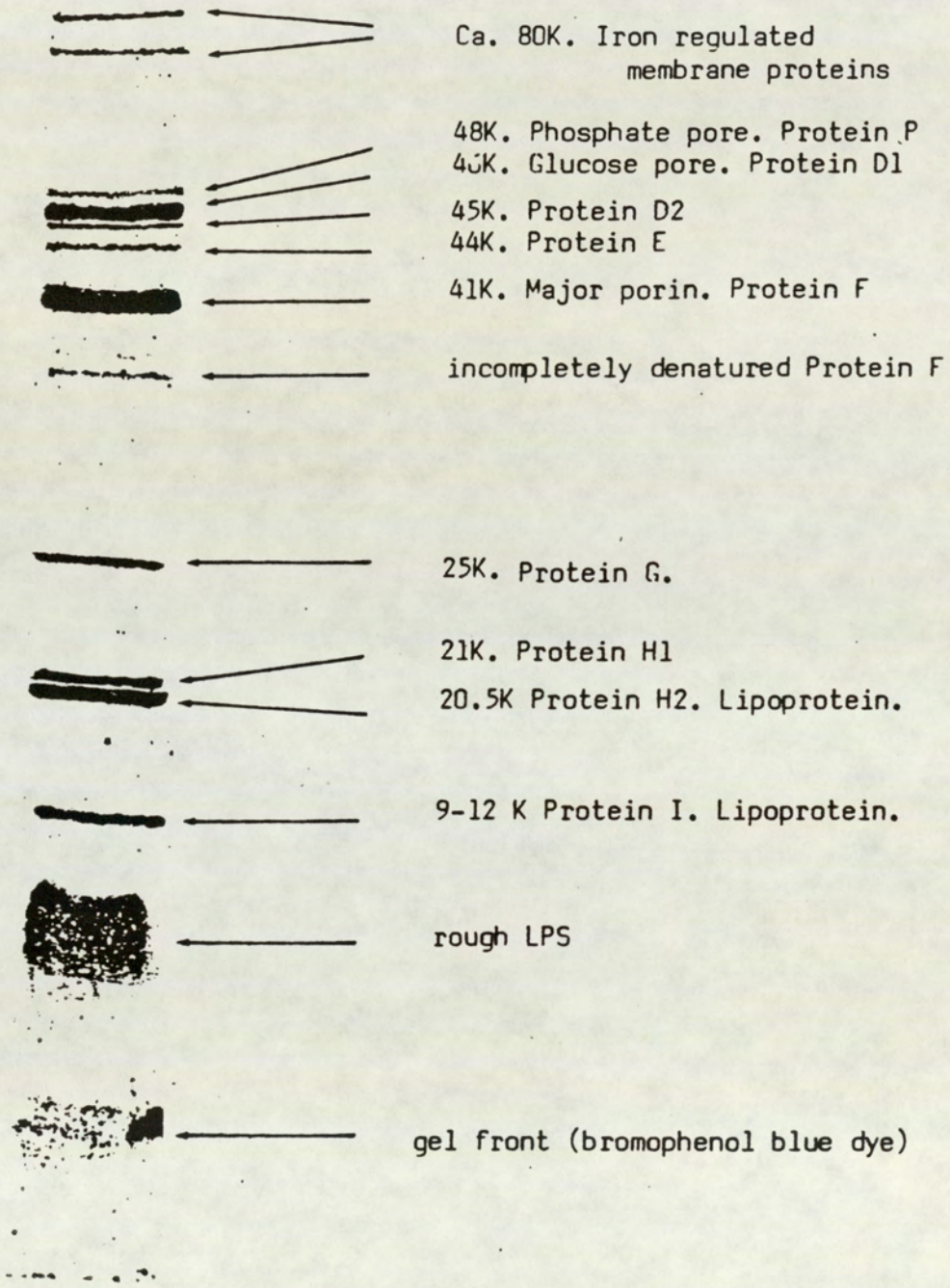


Fig. 1.2. Pattern of OMPs of *P. aeruginosa* following separation by SDS-PAGE (Lugtenberg *et al*, 1984) on 14% acrylamide gels. OM preparation denatured at 100°C in SDS and 2-mercaptoethanol. The approximate molecular weight and function of the proteins are shown where known.

when heated to 70-100°C than at lower temperatures. For proteins D_1, D_2, G and H_1 this implies a rearrangement within the molecules such that the molecular weight appears to have increased and is not due to degradation. The modification can be reversed by addition of LPS as found for the OmpA of *E. coli* K12 (Schweizer *et al*, 1978), hence, the proteins are classed with this type of heat modifiable protein (Hancock and Carey, 1979). Protein F, however, is not included in this classification and requires prolonged boiling in SDS and 2-mercaptoethanol for complete denaturation to the apparent higher molecular weight position. The extensive β sheet structure of this protein (Mizuno and Kageyama, 1979b) may be involved in the modification.

Proteins D_1 and D_2 have very similar molecular weights and have not always been recognized as separate polypeptides (Mizuno and Kageyama, 1978, 1979b). Hancock and Carey (1979) demonstrated that D_1 was induced in cells grown in media containing glucose as the sole carbon source and further showed it to be a porin protein (Hancock and Carey, 1980). Thus, a role in the high affinity glucose uptake mechanism has been postulated for this protein which is co-regulated with a periplasmic glucose-binding protein (Hancock and Carey, 1980). D_1/D_2 is surface-exposed (Lambert and Booth, 1982).

Protein E is also slightly induced after growth of the organism on glucose, although it is generally present in the OM irrespective of culture conditions.

Protein F is the major porin protein of *P. aeruginosa* (Hancock *et al*, 1979) with a molecular weight of about 35 to 37K in its unmodified form and 41K following complete denaturation. The protein is non-covalently linked to PG (Hancock *et al*, 1981) and has been shown to exist in the membrane as an oligomer, probably a trimer (Angus and Hancock, 1983). Lambert and Booth (1982) using radiolabelled lactoperoxidase demonstrated

the surface location of this protein which was further confirmed by Mutharia and Hancock (1983) who used indirect immunofluorescence techniques with monoclonal antibodies to protein F.

A function for protein G has not yet been established but its expression is reduced under Mg- and NH_4^+-limiting conditions and if pyruvate is the carbon source. (Nicas and Hancock, 1980).

The molecular weights (21 and 20.5K) of proteins H_1 and H_2 are very similar and were initially seen only as one band labelled H (Mizuno and Kageyama, 1978, 1979b). Expression of protein H_1 is greatly increased under magnesium limitation (Nicas and Hancock, 1980) as described in section 1.1.2. Protein H_2 is peptidoglycan-associated and is likely to represent the lipoprotein initially described as H by Mizuno (1979).

Protein I is the third peptidoglycan-associated protein of *P. aeruginosa* and is analogous to the Brauns lipoprotein of *E. coli* (Mizuno and Kageyama, 1979a). It is rich in α helical structure (Mizuno and Kageyama, 1979b) and exists in both free and bound forms (Mizuno and Kageyama, 1979a; Hancock *et al*, 1981). The role of lipoproteins is not clear although they are likely to be involved in maintaining the integrity of the OM (Inouye, 1979) and in anchoring it to the PG layer (Sonntag *et al*, 1978).

The expression of protein P and the IRMPs has already been discussed in section 1.1.1.

1.2.1.3. Lipopolysaccharide.

The LPS of most *P. aeruginosa* strains bears some resemblance to *Salmonella* isolates in that the molecule can be subdivided into 3 basic regions a) the hydrophobic, biologically active lipid A, b) a core polysaccharide rich in monosaccharide and phosphate residues and c) an amino sugar-containing, highly variable side region (Wilkinson, 1983). Variations in the side chain length largely account for the marked size

heterogeneity of LPS molecules within a single strain as observed by SDS-PAGE (Kropinski *et al*, 1985).

The lipid A component is based on the 1,6-β-linked disaccharide of D-glucosamine. The amino moeities of the hexosamine are substituted with 3-OH 12:0 residues, whilst 3-OH 10:0, 12:0 and 2-OH 12:0 molecules are ester-linked to the backbone, or to the hydroxy groups of other fatty acids (Fig. 1.3). Other fatty acids often present in low amounts include C16:0 although the precise fatty acid content varies with growth conditions including temperature. Nonetheless, certain of the fatty acids are distinctive of *Pseudomonaceae*. For example, 2-OH acids are rarely found in enterobacterial lipopolysaccharides and 3-OH acids, when present, are C14 acids (Wilkinson, 1983). Another feature of *P. aeruginosa* LPS is the very high phosphorous content, up to 10 residues per molecule distributed between core and lipid A.

The inner core is relatively conserved, comprised mainly of L-glycerol-D-manno-heptose, KDO, phosphate and ethanolamine (Wilkinson, 1983). Additional characteristic components include D-glucose, L-rhamnose, D-galactosamine and L-alanine although proportions of the respective components differ, again due to cultural conditions and, to a degree, between strains. The heptose content, in particular, is known to fluctuate depending on magnesium levels (Day and Marceau-Day, 1982). Phosphate groups in the KDO-lipid A region of the LPS have a high affinity for divalent cations (Mg^{2+} and Ca^{2+}) and by the formation of cross bridges are assumed to be important in maintaining the integrity of the OM (Wilkinson, 1983). *P. aeruginosa* is unusually sensitive to EDTA and this feature is probably a result of chelation of the cations so destabilizing the membrane (Wilkinson and Galbraith, 1975). The proposed stucture for *P. aeruginosa* PAO-1 core polysaccharides is shown in Fig. 1.4.

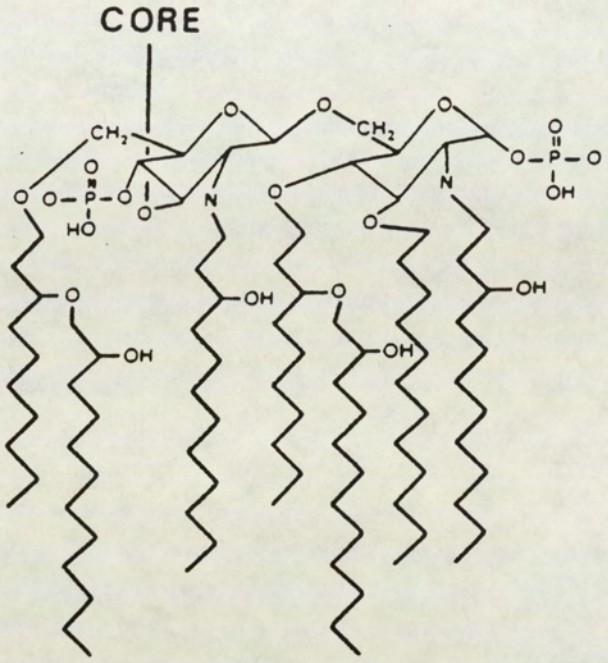


Fig 1.3. Proposed structure for the lipid A of *P. aeruginosa*.

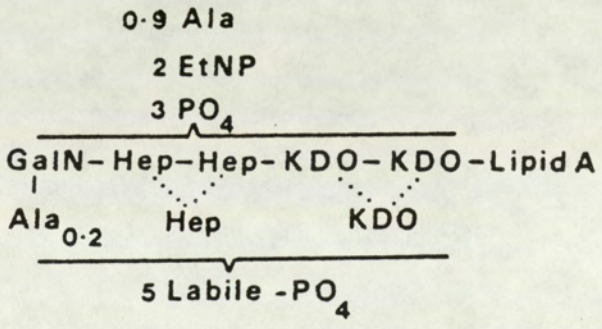


Fig. 1.4. Structure of the core polysaccharides from *P. aeruginosa* PAO-1. Ala = alanine; Hep = heptose; GalN = galactosamine.

From Kropinski *et al* (1985).

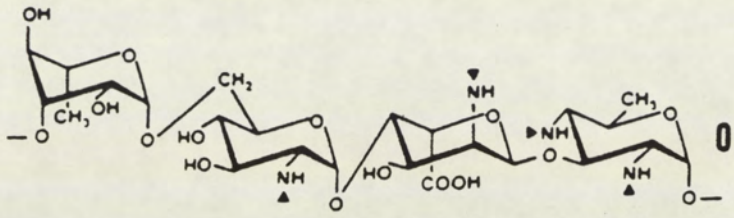
The 'O' antigen regions consist of repeating polysaccharide subunits which are extremely variable and form the basis of the immunological typing system for substrains (O-serotyping; Bergan (1975)). *P. aeruginosa* subunits typically contain unbranched tri- or tetrasaccharides rich in N-acetylated amino sugars. Detailed structural analysis of 'O'-specific polysaccharide chains from a number of different serotypes have now been determined, principally by Knirel and colleagues (Dmitriev *et al*, 1982; Knirel *et al*, 1982, 1986 a and b, 1987), revealing a high proportion of amino sugars and certain sugars unique to the *Pseudomonaceae*. Proposed stuctures for 6 serotypes labelled according to the serogrouping of Homma (1982b) are illustrated in Fig. 1.5.

It is considered that the total LPS population comprises a range of individual molecules differing in the number of O-antigen subunits ranging from 0 to over 40 (Jann *et al*, 1975). However, the proportion of LPS molecules with attached side chains, and the number of subunits in the side chains are dependent, in part, on media composition and growth temperature (Kropinsky *et al*, 1985; Kropinski *et al*, 1987).

Like other bacterial lipopolysaccharides, those from *P. aeruginosa* possess numerous and varied biological properties. Exposed on the surface of the cell, they act as antigens and have known endotoxic activity largely due to the inherent toxicity of lipid A (Lüderitz *et al*, 1973). Furthermore, they provide receptors to some bacteriophages and bacteriocins (Temple *et al*, 1986, a and b) and are directly involved in the uptake of cationic antibiotics. A summary of the pathophysiological effects of *P. aeruginosa* LPS is described by Kropinski *et al* (1985).

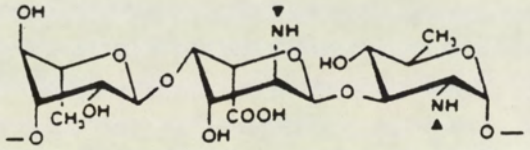
1.2.2. Extracellular polysaccharide.

Exopolysaccharide production by cells of *P. aeruginosa* is under the control of several genes that do not all map in a single chromosome (Fyfe



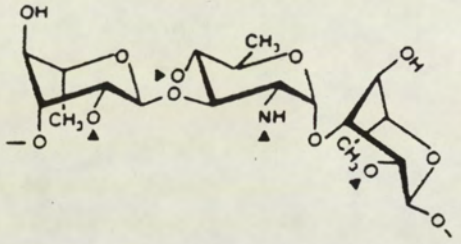
0:1

-3)L-Rha(β1-6)D-GlcNAc(α1-4)L-GalNAcA(α1-3)D-Bac(NAc)$_2$(α1-



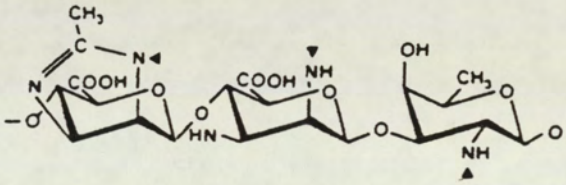
0:2a,c

-3)L-Rha(α1-4)L-GalNAcA(α1-3)D-QuiNAc(α1-



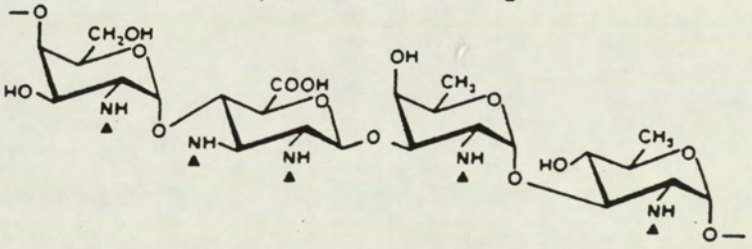
0:5

-3)2-OAc-L-Rha(α1-3)4-OAc-D-QuiNAc(α1-3)2-OAc-L-Rha(α1-



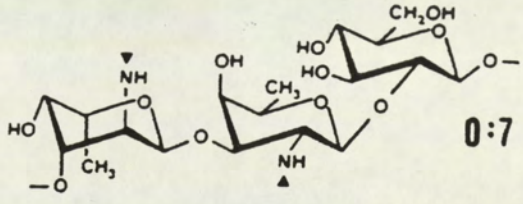
0:3a,b

-4)D-ManImA(β1-4)D-Man(NAc)$_2$A(β1-3)D-FucNAc(β1-



0:6

-4)D-GalNAc(α1-4)D-Glc(NAc)$_2$A(β1-3)D-FucNAc(α1-3)D-QuiNAc(α1-



0:7

-3)L-FucNAc(α1-3)D-FucNAc(β1-2)D-Glc(β1-

Fig. 1.5. Proposed structures for the 'O' side chains of 6 Homma serotypes of *P. aeruginosa*. Rha=Rhamnose; GlcNAc=N-acetylglucosamine; GalNAcA=2-acetamido-2-deoxygalacturonic acid ; Bac(NAc)$_2$=diN-acetylbacillosamine (2,4-diacetamido-246-trideoxyglucose); QuiNAc=N-acetylquinovosamine; ManImA=2,3-(1-acetyl-2-methyl-2-imidazolino-5,4)-2,3-dideoxymannuronic acid; Man(NAc)2 =2,3-diacetamido-2,3-dideoxymannuronic acid; FucNAc=N-acetylfucosamine; Glc(NAc)$_2$A=2,3-diacetamido-2,3-dideoxyglucuronic acid; Glc=glucose.

and Govan, 1980) and the production of such material is conditional on a number of environmental factors (Costerton *et al*, 1983). It is now considered that most strains of *P. aeruginosa* produce exopolysaccharides to differing degrees and of varying composition not, as previously thought, only those isolates described as mucoid by virtue of their colonial morphology on BHI agar (Costerton *et al*, 1983; Chan *et al*, 1984). This was clearly demonstrated by Chan *et al* (1984) who selected 5 strains of *P. aeruginosa*, only 2 of which were classed as "mucoid", and showed that all strains produced large amounts of polysaccharide and grew as mucoid masses in a Mg^{2+} and gluconate-supplemented chemically-defined liquid medium. They further described how conversion of the liquid medium to a solid by the addition of agar inhibited exopolysaccharide production in 3 of the 5 strains indicating that physical factors may also play a role in determining synthesis. The exact nature of the polysaccharides produced appears to be dependent on the strain and the physiological state of the organisms.

One of the exopolysaccharide polymers which has been well-studied is an alginic acid-like, O-acetylated polysaccharide composed of D-mannuronic and L-guluronic acids (Linker and Jones, 1966; Sutherland, 1977). Its copious production by the classic "mucoid" strains, most commonly isolated from the lungs of CF patients, has been long recognized (Evans and Linker, 1973), but a recent report (Anastassiou *et al*, 1987) demonstrated that the extracellular materials from 6 other *P. aeruginosa* clinical isolates classed as non-mucoid (3 from blood cultures and 3 from urine cultures) also contained alginate in varying amounts. The degree of alginate production by different strains under different growth conditions may be due to the repression or loss of activation (or both) of key alginate biosynthetic enzymes and the results of Sá-Correia *et al* (1987) suggest that various levels of repression or activation exist. Neutral sugars and amino sugars

were also evident in the material examined by Anastassiou *et al* (1987) from the isolates described but in higher proportions in the strains termed "non mucoid". A further exopolysaccharide produced by *P. aeruginosa* is a homopolymer of 2-keto gluconate (Chan, 1980; Costerton *et al*, 1981) and its production also appears to be dependent on environmental factors. Most strains additionally synthesize a glycolipoprotein (Bartell *et al*, 1970; Sensakovic and Bartell, 1977) and, polysaccharides which appear to be the high molecular weight form of LPS 'O'-side chains (Pier *et al*, 1978).

1.2.3. Surface appendages.

Flagella and pili are protein structures anchored in the OM. The isolated flagella of *P. aeruginosa* has a molecular weight of approximately 53 to 55K (Montie and Stover, 1983) and each is comprised of a number of repeating polypeptide units called flagellin. The structure as a whole is thread-like and serves to propel the bacteria by its rotating motion. Pili are shorter and straighter than flagella and are usually found at the poles of the cell. Consisting of an 18K polypeptide unit (Frost and Paranchych, 1977; Paranchych *et al*, 1979) they provide attachment sites for bacteriophages (Bradley and Pitt, 1974). They have also been implicated in mediating adhesion of *P. aeruginosa* to each other and to host tissue (Woods *et al*, 1980; Sato and Okinaga, 1987).

1.3. The role of *P. aeruginosa* in infection.

P. aeruginosa has assumed an increasingly prominent role as the aetiological agent in serious infections in hospitalized patients over the past 2 to 3 decades. Its capacity to grow over a wide temperature range in almost any moist surroundings and its non-fastidious nutrient requirements have determined its ubiquitous occurrence in the environment. Nonetheless, it rarely causes infection in healthy individuals and is isolated from the faeces of only 4 to 12 % of the normal population (Bodey *et al*, 1983). However, described as an opportunistic pathogen, it readily causes infections among immunocompromised or otherwise debilitated patients and is responsible for 10 to 20% of infections in most hospitals (Bodey *et al*, 1983; Bryan *et al*, 1983; Neu, 1983). Infections due to *P. aeruginosa* occurred infrequently until after the introduction of sulphonamides and penicillins, when, being notoriously resistant to the action of many antibiotics (Brown, 1975) the organism flourished in sites from which other bacteria were eliminated (Cross *et al*, 1983). It is now the most common pathogen recovered from patients who have been hospitalized for more than one week (Bodey *et al*, 1983).

Especially prone to *Pseudomonas* infections are certain populations of patients outlined in table 1.1. Most of these patients have impaired natural barriers to infection due to injury, trauma or invasive medical techniques, or, alternatively, are immunocompromised with neutropenia (leukaemia and cancer patients receiving chemotherapy) or have altered clearance mechanisms as in cystic fibrosis (CF) patients. Several new groups of people susceptible to infection with the organism have more recently emerged including drug addicts and contact lens wearers (Cross, 1985).

Table 1.1. Patient groups susceptible to *P. aeruginosa* infection.

Condition	Most prevalent infection
Diabetes	Malignant otitis externa
Drug addiction	Endocarditis, osteomyelitis
Leukaemia	Septicaemia, typhlitis
Cancer	Pneumonia, septicaemia
Burn wound	Cellulitis, septicaemia
Cystic fibrosis	Pneumonia
CNS Surgery	Meningitis
Tracheostomy	Pneumonia
Neonatal period	Diarrhoea
Corneal ulcer	Panophthalmitis
Vascular catheterization	Suppurative thrombophlebitis
Peritoneal dialysis	Peritonitis
Urinary catheterization	Urinary tract infection

From Bodey *et al* (1983).

1.3.1. Virulence factors.

From the list in table 1.1 it is also clear that the organism is capable of producing different kinds of disease ranging from acute localized infections (keratitis, panophthalmitis) and chronic localized infections (CF lung disease, osteomyelitis) to acute systemic infections (burn victims and leukaemic patients). Its propensity to cause these diverse conditions, with such deleterious effects in some cases, is in part due to the large number of extracellular and intracellular pathogenic factors specific to this organism (Liu, 1979; Bodey *et al*, 1983).

Extracellular factors include the production of a variety of toxins and enzymes.

Exotoxin A is produced by more than 90% of *P. aeruginosa* clinical isolates (Liu, 1973) and on a weight ratio represents the most lethal extracellular product of the organism (Stephen and Pietrowski, 1981). Investigations have shown that in animals the molecule is 20,000 times as toxic as endotoxin, producing tissue necrosis, neutropenia and death (Liu, 1974). Its mode of action has been elucidated by Iglewski and Kabat (1975) who demonstrated that it inhibits protein synthesis by catalysing the transfer of the ADP-ribosyl moiety of nicotinamide adenine dinucleotide to elongation factor EF-2. The latter is essential to protein synthesis, but coupled to ADP-ribosyl it is non-functional. This effect closely resembles the action of diphtheria toxin (Bodey *et al*, 1983). Also similar to diphtheria toxin, exotoxin A is excreted in an enzymatically inactive form (molecular weight 66K) which is highly toxic to animals and tissue culture cells. It is thought that the enzymatic fraction is "buried" and that alterations in structure permit the active site to be exposed or to assume an active configuration. This can be achieved *in vitro* by treatment of the reduced toxin with urea (Vasil *et al*, 1977), chymotrypsin plus NAD, or chemicals such as cyanogen bromide (Lory and Collier, 1980). The fragments

produced are no longer toxic implying that the remaining part of the molecule may be responsible for delivering the toxin to mammalian membrane receptors and perhaps across the membrane (Pavloskis and Wretlind, 1982). Following elaboration, exotoxin A can enter the circulation where its concentration increases with time. A target organ is the liver where it severely impairs the protein synthetic capacity so creating metabolic abnormalities. This is particularly significant in burn patients whose metabolism is already severely disturbed following the thermal injury (Holder, 1985). Furthermore, exotoxin A may aid penetration of the host's physical defences, as suggested by its necrotizing effects on the skin (Pollack *et al*, 1977). It is also thought to be cytotoxic for human macrophages, preventing phagocytyosis *in vitro* (Pollack and Anderson, 1978), and finally, it inhibits human bone marrow granulocyte-macrophage progenitor cells at a low concentration (Stuart and Pollack, 1982). Despite these numerous deleterious effects, there is a recent report which suggests that neither exotoxin A or the proteolytic enzyme elastase play a major role in the virulence of *P. aeruginosa* in burn wounds. The ability to assimilate iron is considered far more important in this respect (Wretlind *et al*, 1987). Yields of exotoxin A were shown to increase when cultivated in iron-depleted media (Bjorn *et al*, 1978 and 1979) and it seems likely that the effect of iron is at the translation or transcription level and not in the export process since a precursor of toxin A is not synthesized in media with a high iron content (Lory and Tai, 1985). A gene involved in regulation of exotoxin A expression has been cloned (Hedstrom *et al*, 1986) but the cloned fragment does not contain the toxin structural gene.

Exoenzyme S is a second ADP-ribosyl transferase produced by *P. aeruginosa* but, unlike exotoxin A, it does not modify EF-2. Instead it catalyses the transfer of ADP-ribosyl to a number of other proteins as yet uncharacterized (Sokol *et al*, 1981). Nicas and Iglewski (1984) have

demonstrated that loss of exoenzyme S production in burn infections was associated with a very large (2000-fold) loss of virulence. Moreover, in a chronic rat lung model, although exoenzyme-S deficient mutants were able to colonize the lung equally, the pathological damage produced was markedly reduced compared to the parent strain (Nicas and Iglewski, 1985; Woods and Sokol, 1985). Similarly in burned mice, mutant and parent strains proliferated in the wound but only the latter spread to blood and other tissues. It is thus proposed that whilst the enzyme may not contribute to initial colonization, it is important in establishing disseminated infection (Nicas *et al*, 1985). More recently, Woods and Que (1987) succeeded in purifying the enzyme without the use of detergents, so producing a compound which retains its toxicity to animals and tissue cell culture lines. Gel filtration indicated the enzyme molecular weight to be 105K but this may represent aggregates of different molecular species, as seen on SDS-PAGE.

Another toxin of *P. aeruginosa*, cytotoxin (formally known as leukocidin) has a marked destructive action on human PMNs, probably mediated by decreasing the integrity of the PMN membrane following alteration of the phospholipid composition, so causing an influx of Ca^{2+}. This results in leakage of the lysosomal contents into the cytosol (Hirayama and Kato, 1984; Baltch *et al*, 1985) and may consequently have a role in pathogenicity.

P. aeruginosa strains have been reported to produce collagenases, elastase, alkaline protease and nucleases (Liu, 1974) as well as 2 haemolysins, reviewed by Lory and Tai (1985). Proteases, particularly elastase and alkaline protease, have the capacity to cause tissue damage when injected into the lung or eye inducing haemorrhage and necrosis (Kawaharajo and Homma, 1975; Woods *et al*, 1982). Such compounds have a wide range of actions. Elastase can degrade elastin, a major component of several mammalian tissues, and causes dermonecrosis following SC injection of 10μg

purified enzyme, thus demonstrating its destructive potential (Wretlind and Wadstrom, 1977). It has also been found to cleave human type III and IV collagens present in basement membranes and slowly to degrade type I collagen in interstitial membranes (Heck *et al*, 1986). Additionally, it has been implicated in the inactivation of complement-derived chemotactic factors (Schultz and Miller, 1974) and phagocytic PMNs (Kharazmi *et al*, 1984a and b). The latter effect is possibly due to proteolytic cleavage of cell receptors necessary for phagocytosis (Kharazmi *et al*, 1986). A role in the degradation of IgG has been proposed (Döring *et al*, 1981; Holder and Wheeler, 1984) as well as the destruction of PMN elastase provided the concentration of the latter does not exceed that of the bacterial enzyme (Döring *et al*, 1985). The above examples highlight the contribution of proteases, especially elastase, to the success of *P. aeruginosa* in many infection types where they enhance destruction of tissues and host defences so promoting bacterial proliferation and invasion.

A number of pigments are produced by *P. aeruginosa* including chloraphin, pyomelanin, pyorubin, pyoverdin and pyocyanin (Hugh and Gilardi, 1974). These pigments, particularly pyocyanin, may suppress growth of other bacteria so facilitating colonization by *P. aeruginosa* (Bodey *et al*, 1983). Pyoverdin has a well-recognized function in iron-uptake along with pyochelin, both being able to stimulate growth in media containing transferrin and in human serum (Cox and Adams, 1985). The importance of iron assimilation by invading bacteria has been described more fully in section 1.1.1.1 and 1.1.1.2. but it is undoubtedly a major virulence factor of pathogenic bacteria.

Cellular factors determining virulence include the presence of pili which have been found to mediate adherence of the organism to buccal epithelial cells (Woods *et al*, 1980) and more recently to mouse epidermal cells both before and after infliction of burns (Sato and Okinaga, 1987).

Slime polysaccharide may be considered to increase the pathogenicity of the organism since it has a toxic effect on neutrophils (Laharrague *et al*, 1984) and may help mediate attachment to surfaces (Ramphal *et al*, 1987). Moreover it provides a degree of protection from host defences and antimicrobial agents (see section 1.1.3). The role of LPS in virulence is unclear, although high titres of antibodies to this OM component at onset of bacteraemia due to *P. aeruginosa* seem to correlate with improved prognosis in such patients (Pollack and Young, 1979). Young (1972) also suggests that LPS antigens of *P. aeruginosa* have properties enabling the organism to resist phagocytosis, such resistance being neutralized by serum containing specific antibody. In addition, Cryz *et al* (1984b) demonstrated that a mutant deficient in 'O' side chain polysaccharide was serum-sensitive and its ability to establish infection was substantially reduced. On the basis of their results the authors suggest that the 'O' side chain masks complement binding sites present either in the core-lipid A region, or in OM proteins.

A functioning flagellum appears to be a considerable advantage in certain infections including burn wounds where motility may assist the rapid dissemination of bacteria and hence their invasive capacity. McManus *et al* (1980) found that when inoculated onto a burn wound, non-motile mutant strains of the organism were significantly less virulent than were motile control strains. A similar finding was reported by Montie *et al* (1982b).

Thus, virulence of *P. aeruginosa* is multifactorial and enables the organism to successfully proliferate in a variety of sites in the body with the potential to cause serious infection. It seems probable that the relative contribution of the many characteristics described may vary in different disease states (Nicas and Iglewski, 1985).

1.3.2. Clinical Infections.

Detailed reviews on the wide range of infections which are caused by *P. aeruginosa* have been made by Bodey *et al* (1983) and Neu (1983) and the following section serves merely to highlight major areas of the body prone to invasion by this microorganism and will focus to a greater degree on the problem of *P. aeruginosa* in burn patients.

Pseudomonas infections of the eye have been reported for nearly 100 years, the most common form being corneal ulceration. The latter progresses rapidly producing a greenish pus and, without effective treatment, will lead to panophthalmitis. This is partly due to bacterial proteases which destroy the corneal tissue (Ayliffe *et al*, 1966). Infection can follow a minor abrasion or cataract surgery (Fahmy, 1975; Kreger, 1983) and is additionally associated with the use of contact lenses (Cooper and Constable, 1977; Bohigian, 1979).

Ear infections (largely otitis externa) commonly occur in saturation divers who live in pressurized diving bells with high temperatures and high humidity for several weeks (Alcock, 1977; Kenward *et al*, 1984). Malignant external otitis is a severe complication of elderly diabetic patients (Bodey *et al*, 1983). Osteomyelitis of the temporal bone may develop following access of bacteria to the deep tissue of the external auditory canal and the patient presents with severe pain, a purulent discharge and oedema. The high mortality rate of this infection has fallen with improved antimicrobial therapy and surgical techniques to remove debris.

Skin lesions characteristically caused by *P. aeruginosa* include toe-web infection, pyoderma, and ecthyma gangrenosum. The latter develops mainly in the axilla, groin or perianal regions and forms quickly over 12 to 24 h, resulting in a blue/black necrotic region surrounded by an area of erythema. This condition and several other infections of the skin frequently follow *Pseudomonas* septicaemia (Bodey *et al*, 1983).

Drug addicts are a distinct group of people susceptible to certain *P. aeruginosa* infections rarely seen in other patients. These include endocarditis involving the tricuspid valve and often requiring surgical excision (Neu, 1983) and, osteomyelitis of the vertebra and sternum (Holtzman and Bishko, 1971). Osteomyelitis is also a recognized complication of puncture wounds of the foot (Johnson, 1968) in which surgery may be necessary to eradicate the infection (Neu, 1983). Additionally, *P. aeruginosa* infections following joint replacement operations have been reported and this can lead to severe osteomyelitis and cellulitis. Again, this can only be resolved by removal of the artificial joint and debridement of infected bone. In such infections *P. aeruginosa* was shown to be growing in microcolonies in a thick biofilm on the surface of the artificial joint (Gristina and Costerton, 1985).

The incidence of *P. aeruginosa* bacteraemia rose significantly after 1950 and now accounts for between 7 and 18% of all such episodes caused by Gram-negative organisms (Bodey *et al*, 1983). The majority of cases are the result of underlying illness which predisposes to infection, including immunosuppressed cancer patients undergoing chemotherapy or radiotherapy (Hughes, 1983; Bodey, 1985). The risk of infection in neutropenic patients increases substantially if the neutrophil count falls below $1000/mm^3$ (Bodey *et al*, 1966). The prognosis frequently depends on the severity of the existing disease (Curtin *et al*, 1961) and fatality rates have varied from 37 to 77%. This is considerably higher than in septicaemia due to other Gram-negative bacteria (Bodey *et al*, 1983) and these figures rise sharply if correct antibiotic therapy is not instituted immediately. However, introduction of improved anti-*Pseudomonas* penicillins, carbenicillin. ticarcillin, piperacillin and the aminoglycosides have increased survival in cases of septicaemia (Bodey, 1982).

Tracheal bronchitis, pneumonia and lung infection in CF patients are the 3 predominant pulmonary diseases caused by *P. aeruginosa* (Reynolds and Fick, 1980). The first most commonly follows endotracheal intubation, but this condition may resolve without drug treatment if therapy to improve pulmonary clearance is instituted. Pneumonia is a severe complication of cancer patients and patients in intensive care units and mortality rates are high ranging from 50 to 80% (Bodey *et al*, 1983). This may reflect poor penetration of most antibiotics into bronchial secretions. *Pseudomonas* pulmonary infection is frequently associated with CF patients (colonization rates 18 to 80% ; Høiby and Olling, 1977) and whilst it is secondary to the actual disease state it is the cause of 90% of the mortality in such patients (Bodey *et al*, 1983). The bacteria infecting the lung commonly secrete copious amounts of mucoid exopolysaccharide although reasons for the change to mucoid formation are not fully understood (Costerton *et al*, 1979). The mucoid trait appears to be more stable under iron-limitation (Jones *et al*, 1977; Boyce and Miller, 1980 and 1982; Ombaka *et al*, 1983) and, as Brown *et al* (1984) demonstrated that bacteria grew under iron-restriction in the lung environment, this factor may be a selective pressure in favour of mucoid exopolysaccharide production. Following colonization, it is almost impossible to eradicate the organism and, although frequent therapy with high doses of antibiotics improves the clinical picture, microcolonies of *P. aeruginosa* embedded in alginate-like polysaccharide and the thick mucus secretions of the lung remain. CF patients may harbour the bacteria for upwards of 20 years, but despite this, episodes of bacteraemia are rare. This is, in part, due to host immune defences which are essentially intact (Schiøtz, 1982) and which can destroy any free forms of bacteria that might otherwise cause bacteraemia. However, these defences are unable to eliminate bacteria localized in the lung. It is now considered that much of the tissue damage in the CF lung may occur as a result of

immune complex formation (Høiby and Schiøtz, 1982) and it is this destruction that causes the fatal pulmonary insufficiency, cor pulmonale and anoxia seen in CF patients.

P. aeruginosa is one of the predominant pathogens in nosocomial urinary tract infection (UTI) (Turck and Stamm, 1981). Predisposing factors for UTI include anatomical abnormalities leading to urinary stasis (Sobel and Kaye, 1984) and surgical manipulation and catheterization, the latter often only being cured after removal of the infected catheter. This is most likely due to the extensive bacterial biofilms which may develop on the catheter surfaces and which are extremely resistant to the action of antibiotics (Nickel *et al*, 1985 a and b). *Pseudomonas* infections are more complicated than those caused by other Gram-negative bacteria such as *E. coli*, and pyelonephritis may result if bacteria ascend the ureters and reach the kidneys (Sobel and Kaye, 1984). The ability to adhere to catheter material and bladder tissue is a prerequisite for persistent UTI, in order to withstand the continual flushing of the bladder. This may in part be achieved by pili (Woods *et al*, 1980; Sato and Okinaga, 1987) although the observation that non-piliated strains adhere well to rat bladder epithelium indicates that pili-independent mechanisms may operate (Vardi *et al*, 1983).

Pseudomonas infections associated with other implanted prosthetic devices are known to occur, notably with indwelling peritoneal dialysis catheters. Such infections are mostly caused by Gram-positive organisms, predominantly *Staphylococcus aureus* and *S. epidermidis* (Waraday *et al*, 1987). Nonetheless, *P. aeruginosa* is an important pathogen in this respect since resulting peritonitis episodes with this organism are clinically more severe and have a lower cure rate (Krothapalli *et al*, 1982). Peritonitis caused by *P. aeruginosa* has been associated with abscess formation, peritoneal fibrosis and a high failure rate of antibiotic therapy often necessitating removal of the catheter (Gokal 1982; Krothapalli *et al*, 1982).

A recent study demonstrated that both *E. coli* and *P. aeruginosa* multiplied more than 1000-fold in effluents from patients undergoing continuous ambulatory peritoneal dialysis (CAPD) whereas coagulase-negative *staphylococci* and *S. aureus* grew minimally in these fluids (Sheth *et al*, 1986). This may help to explain the severity of Gram-negative infections in CAPD patients. Moreover, in the latter group of patients certain aspects of host defence are compromised. Phagocytosis, a mainline of defence in the peritoneal cavity, is impaired partly due to dilution of available PMNs and macrophages by the large volumes of dialysis fluid (Verbrugh *et al*, 1983). Evidence also suggests that the low pH and high osmolality of dialysis fluids may actually inhibit phagocytic cells (Duwe *et al*, 1981). In addition, peritoneal dialysis effluents from uninfected patients are a poor source of opsonins with low levels of IgG and the C3 component of complement (Clark and Easmon, 1986). These factors all predispose CAPD patients to infection.

P. aeruginosa has also been known to infect the gastrointestinal tract particularly in newborn infants, and a small proportion of meningitis cases are due to this organism (Bodey *et al*, 1983).

1.3.2.1. *Pseudomonas* burn wound infection.

An extensive burn wound is a catastrophic injury. In America more than 2 million people are burned each year, 100,000 of them seriously enough to require hospitalization, and about 10,000 of whom die annually (Gelfand, 1984).

Infection is the major cause of morbidity and mortality in patients with severe burns who survive the initial trauma. It is responsible for between 50 and 75% of all deaths following such injuries (Shires and Dineen, 1982) and in a recent 25 year review of a burn centre in the U.S.A. it was found that the mortality rate due to bacteraemia following burns showed no significant change over the time of study (McManus *et al*, 1985).

The problem in India is even greater in part due to overcrowding and poor nutrition of the patients on admission. In addition, the long delays after burning before patients arrive at hospital increase the chances of infection. Gram-negative sepsis predominantly involving *P. aeruginosa* is a major cause of death in these patients (Jones *et al*, 1978). Graft failures, increased tissue necrosis with conversion of partial thickness wounds to full skin destruction and scarring are all produced as a result of infection. The risk of infection is proportional to the extent of the burn (Yurt *et al*, 1984) and in part reflects the burn-related impairment of host defence mechanisms. Microbial factors are also involved, the invasive capacity of the infecting microorganism being of particular importance.

Following the introduction of effective anti *Staphylococci* antibiotics, Gram-negative bacteria (principally *P. aeruginosa*) emerged as the most serious pathogens. Today, *P. aeruginosa* remains a predominant member of the burn wound flora, its unique aggressiveness and invasive capability playing a vital role in its current prevalence (Pruitt and Lindberg, 1979).

Thermal injury destroys the skin and, consequently, a major organ of host defence. The skin not only acts as a mechanical barrier to penetration through the relatively impermeable keratinized layers, but also, by the formation of fatty acids, may well have an inhibitory action on a number of microorganisms (Speert *et al*, 1980). In addition, the destruction of Langerhans cells, bone marrow-derived immunocompetant cells that migrate to the skin, may further add to the immune dysfunction (Gelfand, 1984). Following destruction of the skin, the denatured protein coupled with the moist warm environment of the burn eschar provides a suitable medium for the growth of microorganisms initially resident, or subsequently seeded onto the wound (Pruitt *et al*, 1983). The blood supply to the wound is reduced as a direct effect of thrombosis of subcutaneous vessels following the burn and as a result of hypotension or shock in the early postburn

period. This partly shields microorganisms from systemically administered antibiotics and from host immunological agents (Order *et al*, 1965).

There is considerable evidence to suggest that both cellular and humoral components of the host defence system are impaired after thermal injury. Reports of depressed serum immunoglobulin levels in the immediate postburn period have been made, IgG being primarily affected (Munster *et al*, 1970). The latter may be related to reduced production of interleukin 2 leading to restricted B cell potentiation by T lymphocytes (Wood *et al*, 1984 and 1986) or to other factors such as increased catabolism and physical leakage of serum proteins from wounds (Munster, 1984).

The complement cascade also undergoes alterations with increased consumption of certain components (Bjornson *et al*, 1977). Suppression of chemotaxis of neutrophils has been described by several researchers (Warden *et al*, 1974; Deitch *et al*, 1982). In some but not all cases, this may be due to the presence of a chemotactic inhibition factor in the serum, as documented in patients following trauma (Christou *et al*, 1980).

Circulatory levels and the function of all cellular elements of the immune system are altered. There is a characteristic early postburn leukopenia and leukocytosis (Eurenius and Brouse, 1973) and considerable evidence now suggests that suppressor T cells are formed 7 to 10 days after injury, which corresponds to the period when post burn trauma sepsis is most likely to occur (Munster, 1976; Miller and Baker, 1979; Lundy and Ford, 1983). Finally, other serum immunosuppressive factors have been convincingly demonstrated by several workers (Constantian *et al*, 1977; Ninneman *et al*, 1982). However, the attempted isolation and characterization of components responsible for this has been difficult (Ninneman *et al*, 1982).

Hence, it is generally agreed that burn injury brings about a suppression of various host responses, although there is less agreement

about which areas of suppression are the most important in terms of infection (Munster, 1984).

Infections of the burn wound can be broadly divided into 2 categories. Non-invasive wound infection is considered to be an infection limited to the burn eschar or exudate. It is characterized by a rapid separation of the eschar and an increased or heavy exudation of purulent material from the wound. Systemic manifestations include a mild to moderate spiking fever and a leucocytosis. Cultures of the eschar or drainage often show 1,000,000 organisms or more per gram of tissue but bacteria are not seen invading surrounding viable tissue. Invasive wound infection may be characterized by a drying of the wound surface with formation of crusts and necrotic tissue, and indicates that spread of bacteria into viable tissue beneath or around the wound has occurred. This can be confirmed by histological examination of wound biopsies. The onset may be sudden, but more often follows evidence of purulent discharge and fever. Partial thickness wounds rapidly develop to full thickness and the patient becomes hypothermic and non-responsive. If these processes are not detected early and vigorous therapy instituted immediately then septicaemia and death may subsequently follow (Alexander, 1979).

A delicate balance, therefore, exists between host defences and the invasive capacity of the microbiological population on the wound, and this determines whether invasive wound sepsis occurs.

The number and density of microorganisms present on the burn wound changes with time after injury, as does the depth of microbial penetration of the wound eschar (Pruitt and Lindberg, 1979). Immediately postburn relatively few organisms colonize the wound and those that do are mainly Gram-positive bacteria. These are gradually superceded by the Gram-negative opportunists particularly *P. aeruginosa* which, for the reasons outlined in section 1.3.1, has a greater propensity to invade (Pruitt *et al*, 1983). At a

major burn centre in Texas, 60% of patients were found to harbour *P. aeruginosa* by the 5th day following burn injury (Pruitt and Lindberg, 1979). It appears that most of the infecting bacteria are nosocomial organisms from burn patients previously on the ward. It has been demonstrated that only 15 to 25 % of *Pseudomonas* colonizing wounds arose from the patients' lower gastrointestinal tract (Sutter and Hurst, 1966).

Wound infections where *P. aeruginosa* is the causal organism are characterized initially by development of a green, foul-smelling discharge over a 2 to 3 day period. As the infection advances the wound may become dry with a green exudate due to pyocyanine production by the bacteria, with patchy areas of dark brown necrotic tissue (Alexander, 1979) and prognosis at this stage is poor. Subsequent bacterial invasion of the blood stream is associated with a mortality of 77% (McManus *et al*, 1985).

Thus, the picture that emerges when *P. aeruginosa* colonizes the wounds of severely burned patients is one of an opportunistic and highly virulent pathogen capable of producing numerous toxic extracellular products. These may promote separation and lysis of the eschar, causing tissue damage and so facilitating invasion of the bacteria into surrounding viable tissue. Dissemination may also be assisted by means of a functioning flagellum. In this mode, the individual mobile bacteria are susceptible to the bactericidal and opsonic actions of antibodies directed against their surface antigens including type-specific LPS, pili, flagella and OM proteins. They are also subject to phagocytosis and the adverse effects of antibiotics at whatever concentration can be maintained in the burn vicinity. These factors are all crucial in preventing the bacteria from subsequently invading the general circulation with the devastating consequences this would entail.

1.4. Host responses to infection.

Host defences against infection can be divided broadly into 2 major mechanisms: specific and non-specific. Non-specific defences encompass a wide range of factors aimed at preventing entry of microorganisms into the host, including the barrier function of the skin, the acidity of gastric juices and the protective actions of secretions bathing surfaces such as the conjunctiva of the eyes and the lungs. Additional mechanisms act to present microorganisms which succeed in gaining entry to body sites, with an environment hostile to their proliferation. This is achieved, in part, by reducing the level of iron available to bacteria (section 1.1.1.2), as well as by production of a spectrum of serum components which serve as mediators of inflammatory reactions. Specific immune defences rely on the recognition of invading microbial antigens and the coordinated response thereof, of antibodies, phagocytic cells, chemotactic factors and complement. Such defences are aimed at promoting the uptake and killing of bacteria and inhibition of the toxic properties of bacterial components and exoproducts.

1.4.1. Humoral immunity.

One arm of the specific immune response involves activation of B lymphocytes, so called because in chickens they are derived from the Bursor of Fabricius (this structure is not present in humans). Prior to stimulation, these cells populate peripheral lymphoid tissues in a resting state but on challenge with antigens which bind to particular receptors on the lymphocyte membrane, a complex sequence of events is initiated, involving cellular differentiation and extensive protein, RNA and DNA synthesis. About 10^5 antibody-like receptors per B cell exist which are formed spontaneously. Hence, when almost any antigen enters the body for the first time there will be a few B cells that react with it specifically. This is followed by a series of mitoses to form a clone of cells which

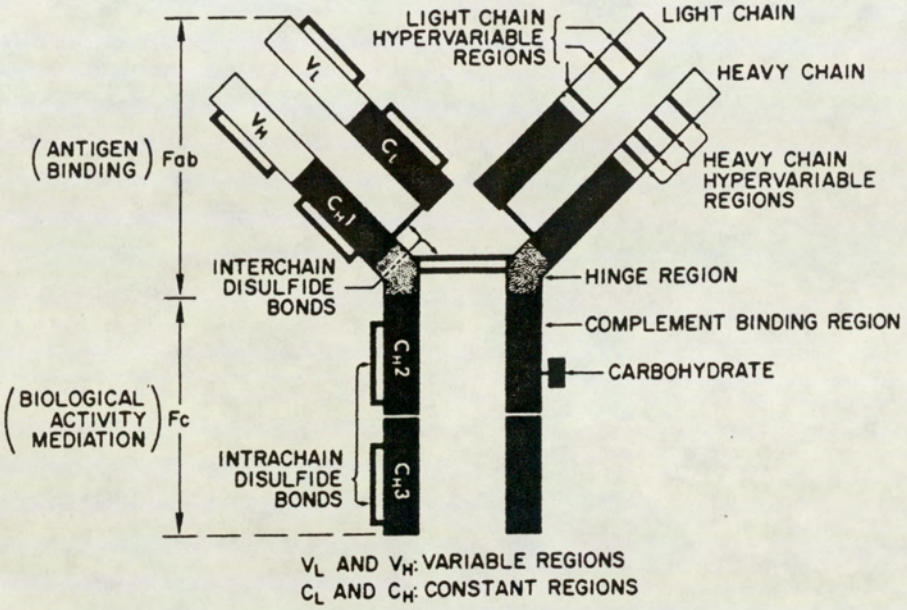
subsequently mature to plasma cells programmed to synthesize and secrete antibodies capable of reacting with the inciting antigen.

The antibodies formed include representatives from the 3 main immunoglobulin classes, IgG, IgA and IgM. A single bacterial cell may have numerous surface antigenic sites such as LPS, pili, OM proteins, each with possibly several epitopes which, in turn, stimulate the formation of a different antibody. The antibodies themselves have varying affinities for their respective antigens and not all are protective. Nonetheless, there is ample evidence that production of antibodies to *Pseudomonas* cell wall components and toxins increases in infection and correlates with survival. Young and Armstrong (1972) suggested that opsonizing antibody was augmented in the sera of patients surviving *Pseudomonas* bacteraemia, whilst Pollack and Young (1979) further linked high acute serum antiexotoxin A and anti LPS titres with improved prognosis in bacteraemia, protection being both independent and additive. High anti exotoxin A titres also correlated with good recovery from infection in a study by Cross *et al* (1980).

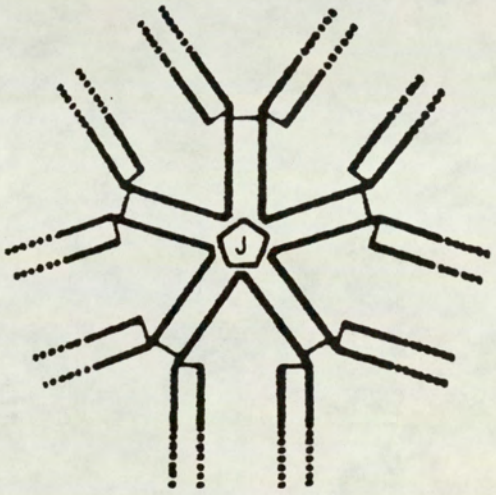
IgG is the most abundant immunoglobulin in the serum of all higher vertebrates. It can also cross the placenta and is present in extravascular tissues, its concentration in such fluids increasing in response to inflammation. It has a molecular weight of 150K and is composed of 2 heavy (γ) and 2 light (κ or λ) polypeptide chains held together by disulphide bonds in the form of a Y (Fig. 1.6, i). Edelman and Gall (1969) proposed that the IgG molecule can be divided into 4 domains, VH, CH1, CH2 and CH3 (Fig. 1.6, i) each serving distinct biological functions, but acting in an overall coordinated fasion. CH2 and CH3 together form the Fc region of the molecule which has no antigen-reactive sites, but complement fixing activity has been demonstrated in the CH2 domain (Isenman *et al*, 1977). The latter only occurs following binding of IgG to the antigen which suggests that conformational changes within the CH2 domain may occur thus exposing

Fig. 1.6. Structure of IgG (i), IgM (ii) and IgA (iii).

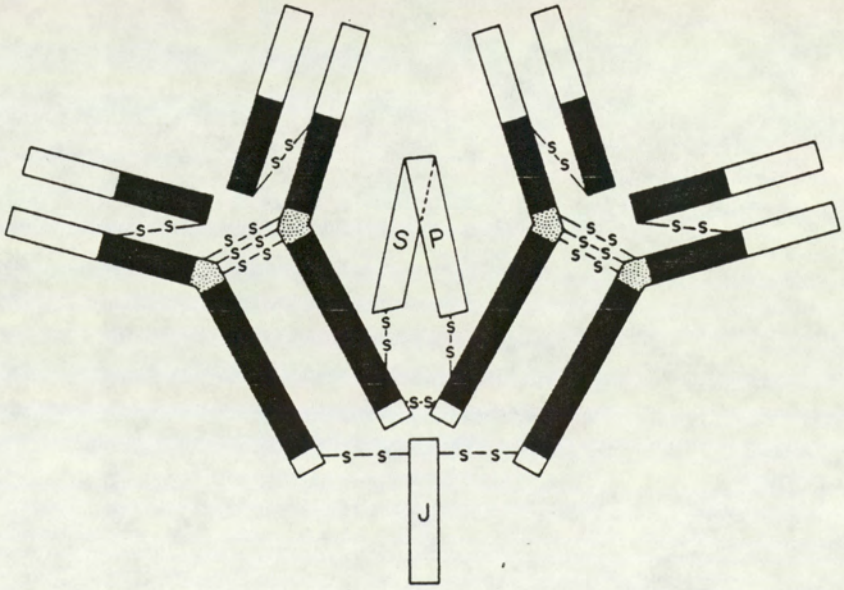
6.1, i.



6.1, ii.



6.1, iii.



the binding site for the first component of the complement cascade (section 1.4.2). The binding site for macrophages has been located in the CH3 domain (Yasmeen *et al*, 1973) and that for PMNs in the Fc region as a whole. The variable regions (VH) comprised of heavy and light chains form the antigen reactive ends with a unique amino acid sequence for a given antibody molecule and are responsible for its specificity. IgG has been divided into 4 subclasses differing by approximately 5% in the sequence of their γ-chains (Siegelman and Capra, 1980). However, the clinical relevance of each of the subclasses in man is only just being realized.

IgM is a polymer of 5 subunits, each with the basic 4-chain structure but with a different heavy chain (μ). It is a large molecule (molecular weight 900K) and as such is mainly confined to the vascular system. It is formed early in the immune response and is very powerful with respect to its avidity and complement activating capacity, bearing 5 times the number of Fc and Fab sites as IgG (Fig 1.6, ii).

IgA circulates in the plasma as a monomer like IgG but is secreted onto mucosal surfaces as a dimer held together by 2 proteins called the J chain and the secretory piece (Halpern and Koshland, 1970; Roitt, 1974) so forming a molecule of molecular weight 385K (Fig. 1.6, iii). The functions of these proteins may be to increase stability of the IgA molecule so rendering it less susceptible to the hostile environments of the gastrointestinal and respiratory tracts. It is thought to have little ability to activate complement, except in aggregate form by the alternative pathway (Feinberg and Jackson, 1983).

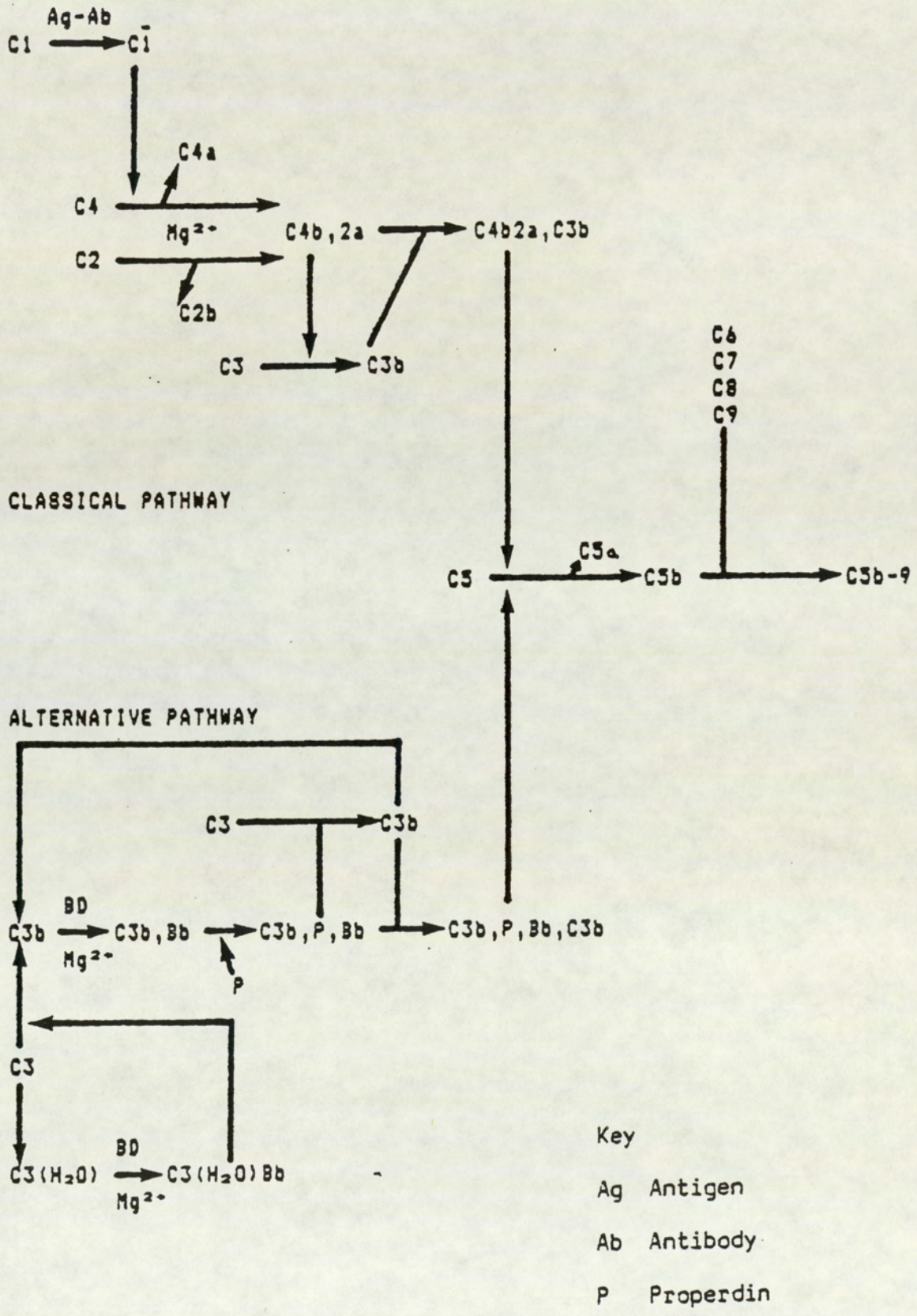
The main antimicrobial actions of antibodies include promoting phagocytosis by acting as opsonins, preventing attachment of microorganisms to surfaces by binding to bacterial appendages involved in adhesion, reducing motility by inhibition of flagella antigens and possibly restricting uptake of essential nutrients by binding to certain OM

receptors. In addition, antibodies directed against microbial toxins may neutralize the effects of such compounds. Finally, by combining with microbes, antibodies activate complement so inducing an inflammatory response ultimately resulting in cell lysis (Mims, 1982).

1.4.2. Complement.

The complement system is characterized by a series of 11 or more proteins which react in an orderly sequence so amplifying immune reactions including inflammation and chemotaxis, and finally causing the destruction of invading microorganisms (Taylor, 1983; Feinberg and Jackson, 1983). The classical pathway of the complement sequence is initiated when the first component C1 binds to IgG or IgM antibodies following their formation of an immune complex with invading microorganisms. C1 circulates as a Mg^{2+} and Ca^{2+} - dependent complex composed of 3 subunits C1q, C1s and C1r. C1q has 6 spatially separated binding sites and binds to Fc portions of antigen-complexed antibody. More than one Fc must bind before activation of C1r and C1s takes place. After binding of C1q to the Fc region, C1s acquires esterase activity and brings about the cleavage of C4 and C2 into C4a and b and C2a and b respectively (Fig. 1.7). In the presence of Mg^{2+} C2a and C4b combine on a cell membrane to form C4b2a which splits C3 into C3a and b. C3a degranulates mast cells producing local inflammation and attracts neutrophils. C3b has opsonic properties and further binds to cell membranes complexing with C4b2a to form C4b2a3b. This molecule is a convertase for C5 cleaving it into C5a which is chemotactic and anaphylactic, and C5b. The latter complexes with C6 and C7 and together they attach to a membrane (preferably bacterial). C567 is chemotactic for neutrophils but also binds C8 and up to 6 C9 molecules, the resulting complex (membrane attack mechanism) acting as a catalyst to destroy the membrane and lyse the cell (Koski *et al*, 1983). Since a single molecule of

Fig. 1.7. Schematic representation of the Classical and Alternative pathways of complement. From: Taylor (1983).



activated C1 generates thousands of molecules of the later components, the whole series is an amplification process and is sometimes termed the complement cascade.

An alternative pathway which does not require antigen/antibody immune complexes may be activated by compounds such as bacterial endotoxin and polysaccharide in conjunction with a number of endogenous plasma proteins, factors B, D and P (properdin) as shown in Fig. 1.7. The end result is production of further compounds capable of cleaving C5, so converging with the classical pathway.

Clearly certain mechanisms are required to prevent uncontrolled activation and complete consumption of the complement components. A C3b inactivator acts, in the presence of a cofator β1H, converting C3b into an inactive form and so preventing total destruction of C3. Furthermore, particular serum proteins inactivate C1 and the anaphylactic fragments C3a and C5a (Feinberg and Jackson, 1983).

1.4.3. Cell-mediated immunity.

The other group of lymphocytes which are activated by recognition of specific antigens are the T cells derived from the thymus. Upon stimulation, they mature to form groups of cells performing distinct functions. Although host resistance to *P. aeruginosa* depends mainly on humoral immune phagocytic action (Homma, 1982a), the importance of T cell-mediated immunity is being increasingly recognized, and is largely due to the cooperation of T cells in many B cell functions. The cells responsible for this are termed T helper cells and they signal B cells to differentiate and produce antibodies. Effective antibody responses to most antigens depend on T helper cooperation (Mims, 1982). Further control of B cell function is mediated by T suppressor cells which act to regulate the magnitude of both cell-mediated and humoral immune responses. Another group of T cells

elaborate soluble mediators or lymphokines with a variety of functions. For example, chemotactic factor attracts macrophages to the site of antigen deposition, migration inhibition factor localizes the phagocytes and macrophage activation factor stimulates the macrophages so increasing their killing capacity. An additional lymhokine termed interleukin 2 in turn stimulates T cell proliferation (Smith *et al*, 1980). Finally, cytotoxic T cells are capable of directly killing certain target cells including virally-infected or tumour cells. T cell-mediated killing of intracellular bacteria has been well-characterized and is thought to be mainly due to macrophages activated by T cell lymphokines. A recent paper by Markham *et al* (1985) using *in vitro* studies suggests that direct killing of extracellular bacteria, including *P. aeruginosa*, can occur with T cells, but the mode of action differs from that of intracellular bacteria. The killing process involves the action of lymphokines on the bacteria themselves. This effect was further promoted by interleukin 1, a hormone synthesized and released by macrophages within a few hours of invasion by bacteria. Interleukin 1 has many other diverse activities which have been well-reviewed by Dinarello (1984) and which play a vital role in numerous immunological responses.

1.4.4. Polymorphs and macrophages.

Polymorphonuclear leucocytes (PMNs) arise in the bone marrow and are characterized by a segmental nucleus and granular cytoplasm. They are continually discharged in vast numbers into the blood but carry out their phagocytic activities in the tissues at sites of inflammation. They only live for a few days but their loss is balanced by entry into the blood from the bone marrow (Mims, 1982). The 3 classes, neutrophils, basophils and eosinophils are distinguished by the staining reaction of their cytoplasmic granules. Of these, neutrophils predominate and are the most important with

respect to microbial destruction. They respond to chemotactic factors including certain complement components (C3a and C5a) and lymphokines. Their major role is the uptake and subsequent killing of invading microorganisms. This firstly requires attachment of particles to the cell membrane of the PMN via an energy-independent process, involving either non-specific or specific ligand-receptor interactions. Several well-defined recognition sites occur in phagocytic cells (Stendahl, 1983) which act as receptors for the Fc moiety of IgG and for activated forms of complement. The latter (CR1 and CR2) are known to be functionally independent of the Fc receptors. PMNs also recognize and bind several other ligands for example carbohydrate residues, mannose, N-acetyl-glucosamine, glucose and galactose. Various bacterial adhesins with lectin-like properties also promote attachment of certain microorganisms to the phagocytic cells (Jones, 1977). Such lectin-like interactions promote strong attachment, however, they are less potent than complement or IgG at initiating ingestion. Whether attachment leads to ingestion or not may be more dependent on underlying properties of the bacteria including the presence of LPS and extracellular polysaccharide (Horwitz and Silverstein, 1980).

Nonspecific recognition is based on the physicochemical properties of both the bacterium and the phagocytic cell. The main properties are negative charge and hydrophobicity (Van Oss, 1978). Less hydrophobic cells or cells with increased negative charge have been shown to impair phagocytic recognition (Van Oss, 1978). It is known that phagocytosis may take place in the absence of specific antibody or complement, but this process is greatly facilitated by the presence of such opsonins bound to microbial cells or antigens. The latter may act to increase the hydrophobicity of the bacteria so additionally enhancing their engulfment by phagocytic cells.

Interaction of a particle with a phagocyte triggers several events which ultimately result in ingestion of the particle, degranulation and a burst of oxidative metabolism. The latter is characterized by increased oxygen consumption, stimulation of the hexose monophoshate shunt (Sbarra and Karnovsky, 1969), activation of the membrane-associated NADPH-dependent oxidase (Cohen *et al*, 1980) and hence, generation of reactive oxygen-derived metabolites (superoxide, H_2O_2, oxygen radicals) (Root and Cohen, 1981). The formation of these oxidising species is accompanied by a flash of light called native chemiluminescence and can be enhanced by adding luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedrone) in biological fluid. This forms the basis of chemiluminescence as a means of measuring the phagocytic process, the light emitted being measurable at a wavelength of approximately 425nm (Easmon *et al*, 1980).

Ingestion is achieved by circumferential flow of pseudopods around the particle forming a phagosome, and requires activation of a calcium-dependent system involving actomyosin microfilaments. Killing of the bacteria is achieved by fusion of lysosomal granules with the phagososme and release of their contents. These include enzymes such as lysozyme, peroxidase, alkaline phosphatase and acid phosphatase among others, as well as lactoferrin (Stossel, 1974 a and b) which, together with the active metabolites of oxygen generated as described, destroy ingested bacteria.

Functional granulocytes are paramount in preventing *P. aeruginosa* infection, granulocytopenia associated with malignancy or abnormalities of existing granulocytes as seen in leukemia predisposing to infection in such patients (Bodey *et al*, 1983; Bodey, 1985). However, the cost and risks of granulocyte transfusion may outweigh the benefits (Klastersky, 1983).

Macrophages are mononuclear cells with a single indented nucleus which have many crucial functions in the mounting of a successful immune response by the host. It seems that separate subpopulations of macrophages carry out

different actions which include phagocytosis, antigen presentation and release of certain controlling compounds. Fixed macrophages line the blood sinusoids of the liver, spleen, bone marrow, adrenals and lymph sinuses, while many are found in the peritoneal and pleural cavities. Hence, they are strategically placed throughout the body to encounter any invading microorganisms. Such cells can "process" microbial and other antigens so presenting them to immune-reactive B and T lymphocytes. They achieve this by holding antigens on their surface in close association with the Ia antigens (Mims, 1982).

One of their major functions is phagocytosis of invading bacteria. The processes by which this is achieved are basically similar to those described for the PMNs although there are certain differences. Macrophages live longer than PMNs and have the synthetic machinery to renew the lysosomal enzymes used to effect killing of ingested bacteria. The enzymes themselves differ in some respects, macrophages not containing oxidising compounds and, in some ways, the bactericidal activity of these cell is inferior to that of the PMNs. However, they have an essential role in phagocytosing bacteria which have escaped the circulating blood phagocytes, their location being ideal for such a function.

As mentioned in section 1.4.3. macrophages also synthesize and release the 15K hormone interleukin 1 whose many actions have only more recently been recognized. Briefly they include shifting iron from plasma to storage, adjusting the temperature set point to >38.5°C, releasing granulocytes from the bone marrow storage pool and granules from neutrophils, as well as inducing varied changes in the biosynthetic activity of hepatocytes (Dinarello, 1984).

To circumvent some of the many facets of the host immune defences outlined above, *P. aeruginosa* has developed certain strategies discussed more fully in section 1.3. These include evasion of phagocytosis. Somatic

and extracellular products of *P. aeruginosa* interfere at all stages of the phagocytic process (Peterson, 1980) and mucoid strains resist the action of phagocytes even in the presence of specific antisera (Baltimore and Mitchell, 1980). Slime glycolipoprotein from this organism is toxic to granulocytes (Sensakovic and Bartell, 1974) and leukocidin (cytotoxin) kills phagocytic cells (Homma, 1982 a). Additionally, *Pseudomonal* elastase has a destructive effect on immunoglobulins (Döring *et al*, 1981) and complement. Of the latter components, only C4 and C7 are resistant to degradation by the enzyme (Schultz and Miller, 1974). In short term assays, most strains of *P. aeruginosa* are resistant to the bactericidal effects of serum (Taylor, 1983). However, growth phase and culture conditions are known to have a marked effect on sensitivity to serum killing and complement action (Taylor, 1984; Anwar *et al*, 1983b).

1.5. Immunological prophylaxis of *P. aeruginosa* infection in burns.

One of the earliest attempts at the immunotherapy of *P. aeruginosa* infection was made nearly 100 years ago when Charrin and Roger (1889) repeatedly administered small doses of living cultures of the organism to rabbits and demonstrated that they became refractory to infection. At this time *P. aeruginosa* was not a major cause of serious disease. However, with its increasing importance as an opportunistic pathogen in such a variety of infections, and particularly with recognition of its resistance to even the most aggressive antimicrobial treatment, a considerable impetus towards developing immunotherapeutic measures to combat the organism has arisen. Evidence that patients developing severe or fatal infections are antibody deficient and that high titres of antibodies to certain bacterial components or exoproducts (LPS and exotoxin A) have correlated with increased survival in many groups of patients (Pollack and Young, 1979) has further prompted the search for successful vaccines. These may have particular value in burn patients to prevent the rapid progression of invasive wound sepsis with its poor prognosis. In these patients *Pseudomonas* infection does not usually occur in the first few days post burn so providing a lag period during which time vaccination may be feasible (Pennington, 1979). To be successful, components of the vaccine would need to elicit high titres of protective antibodies at an early stage post burn, preferably without toxic side effects. Unfortunately, the well-characterized immunosuppression occurring after thermal injury may negate some of the benefits of active vaccination and perhaps points to the potential of passive immunotherapy in burn victims. Nonetheless, a number of active immunization protocols have been tested in such patients with a certain degree of success.

1.5.1. Current *P. aeruginosa* vaccines tested in humans.

Most of the *P. aeruginosa* vaccines presently available are based on whole cell extracts or multicomponent toxoid vaccines and are associated with varying efficacy and levels of toxicity. Certain of the earlier studies, however, involved too few patients or poorly designed trials such that definite conclusions concerning the value of the vaccine preparations could not be made.

Two studies in the late sixties and early seventies involved whole cell vaccines. Feller and Pierson (1968) using only 1 strain of *P. aeruginosa* demonstrated a lower mortality (11%) in burn patients receiving the heat-killed cells along with sera raised against the vaccine strain compared to 40% in historical controls. Nonetheless, such differences may have been due, in part, to improved patient management over the 8 year study period. Sachs (1970) administered a hexavalent preparation and reported a reduction in mortality in patients who received more than 3 vaccine doses compared to historical controls. However, the number of patients tested was low.

Later investigations employed multivalent preparations of more highly purified antigenic extracts. The first of these, a heptavalent vaccine (Pseudogen), was originally described by Hanessian *et al* (1971) and is now marketed by Parke Davis and Company. It is comprised of phenol extracts of 7 Fisher type strains (Fisher *et al*, 1969) thought to represent some 90 to 95% of *Pseudomonas* serotypes encountered in many hospitals. Chemical analysis has revealed that LPS is the primary component. The vaccine was tested in several patient populations at high risk of *Pseudomonas* infection including immunosuppressed cancer patients in which limited protection was evident (Young *et al*, 1973) and CF patients, where no clinical value was demonstrated (Pennington *et al*, 1975). In the latter group, high titres of antibodies have been observed without vaccination and, furthermore, alveolar

damage may be partly due to immune complex formation casting doubts on the potential of any form of vaccination in these patients (Høiby and Schiøtz, 1982). Tests of Pseudogen in burn patients using a variety of immunization protocols did result in more favourable results in that mortality due to *P. aeruginosa* septicaemia was reduced. However, historical controls were once more used to evaluate the significance of the protection (Alexander and Fisher, 1974). It appeared that the major immunoglobulin produced was of the IgM subclass which is known to have a shorter half life and possess less protective capacity than the IgG class of immunoglobulins (Bjornson and Michael, 1970). In all studies, use of the vaccine has consistently been associated with local pain and systemic reactions such as fever, headaches and malaise most likely due to the high LPS content (Young *et al*, 1973; Pennington *et al*, 1975).

A second polyvalent extract vaccine was developed by Miler *et al* (1977) of Wellcome Research Laboratories (PEV-01). Viable cells of 16 *P. aeruginosa* serotypes were extracted with EDTA-glycine for 2 minutes at 37°C with the aim of producing a less toxic preparation. Many experimental animal models demonstrated the protective nature of this vaccine (Miler *et al*, 1977; Pennington and Miler, 1979) which was subsequently tested in human trials. Poor results were again obtained in CF patients which is perhaps not surprising (Langford and Hiller, 1984). In a study conducted with burn patients in a Birmingham hospital little difference in mortality between vaccinated and control groups was observed (Jones *et al*, 1979). However, none of the deaths were attributed to *P. aeruginosa* in this trial. More encouraging data was forthcoming from a study in India. In vaccinated burn patients mortality was 6.6% with no deaths due to *P. aeruginosa* septicaemia compared to 6 in unvaccinated adults and an overall mortality of 40.6% (Jones *et al*, 1979). In this trial no topical wound therapy was instituted, a procedure which is routine in Western burn units (Pruitt *et*

al, 1983). Additionally, immunoglobulin from the plasma of immunized volunteers prevented deaths in a group of burned children compared to a mortality of 21% for controls. However, combined vaccine and immunoglobulin treatment gave less protection than either treatment alone (Jones *et al*, 1980). The cumulative findings of subsequent trials in India yielded similarly encouraging results (Roe and Jones, 1983). The vaccine is thought to be better tolerated than Pseudogen although no direct comparisons have been made. Recently, elegant studies were undertaken by MacIntyre *et al* (1986 a and b) to determine the nature of the antigen responsible for the protection observed with the PEV-01 vaccine. They demonstrated that LPS is a major constituent of at least 15 of the 16 extracts with small amounts of a 16.2K protein not detected in isolated OMs and not thought to represent pili (MacIntyre *et al*, 1986a). Antisera to the vaccine further recognized flagella and OMPs F and H_1/H_2 as minor components. Using an extract from serotype 0:6 evidence was obtained that protection was entirely attributed to the LPS moiety, protein components playing little role in this respect. Furthermore, LPS molecules with more than 10 'O' antigen side chains were the more effective immunogens (MacIntyre *et al*, 1986b).

A small number of human burn patients have been immunized with a multicomponent vaccine consisting of toxoids of elastase, protease and exotoxin A with the protein cell wall antigen, original endotoxin protein (OEP). In this study relatively favourable results were obtained, the vaccine reportedly reducing the incidence of mortality to *P. aeruginosa* (Homma, 1982a). Nonetheless, larger studies are required before definitive judgements on its efficacy can be made (Homma, 1982a).

Recently, human trials with a vaccine "Pyoimmunogen" were carried out in which volunteers were immunized and their plasma used to treat patients with *P.aeruginosa* infections (Krokhina *et al*, 1985). Two types of vaccine were tested, the first prepared under laboratory conditions using 5

strains, the second, under production conditions using 3 strains. Essentially the vaccine is based on water-soluble, heat-stable antigens prepared from cells which are autoclaved at 120°C for 30 min and incubated with 0.25% phenol for 6 weeks. Whole cells are then removed by centrifugation and the supernatant filtered (Stanislavsky *et al*, 1985). The preparation was found to have little toxicity in human volunteers and demonstrated potential in the passive immunotherapy regimen. Low molecular weight proteins or peptides of the cell wall, possibly the OM, are considered to be the key antigens involved in protection, although this has not been directly determined. LPS was thought to be present in very low amounts, however, antibodies to *P. aeruginosa* 'O' antigens were evident in the sera of vaccinated individuals and, as such, it cannot be ruled out that the protection afforded was partly due to the anti-LPS antibodies.

Passive immunotherapy has been advocated in the treatment of *P. aeruginosa* infection to prevent septicaemia. An early study in humans included use of J5 antisera (raised in volunteers against the core glycolipid derived from the J5 mutant of *E. coli* 0111). Enhanced survival in *P. aeruginosa* septicaemia has been linked with high levels of circulating antibody to *E. coli* endotoxin core (Pollack *et al*, 1983a) and, passive therapy of Gram-negative bacteraemia with J5 antisera was reported to increase the recovery rate of severely ill bacteraemic patients in shock from 29 to 82 % (Ziegler *et al*, 1978). *P. aeruginosa* was the second most common infecting organism in this trial although specific data on the efficacy of the antisera in *Pseudomonas* infection has not been determined. Indeed, the protective effect of such therapy has not been observed by all investigators (Pennington and Menkes, 1981). A more recent re-evaluation of J5 antisera was undertaken by Trautman and Hahn (1985) in an animal model but their results were also discouraging with respect to *P. aeruginosa* infection. The data indicated that prevention of lethal Gram-negative

infection was not superior to that of pre-immune serum except in the case of *E. coli* septicaemia. The debate over the benefits of J5 antisera will doubtless continue.

Finally, although protection studies as such have not been performed , the human immune response to high molecular weight polysaccharide (PS) from the slime and culture supernatants of immunotype 1 (IT-1) was investigated by Pier (1982a) and Pier and Thomas (1983), and from immunotype 2 (IT-2) by Pier and Bennet (1986). In both cases side effects were minimal and IgG and IgA antibodies were the predominant immunoglobulins elicited (IgM were also produced with IT-1). The level of antibodies attained was equal to or higher than that found in acute-phase serum from patients surviving *P. aeruginosa* sepsis and, the immunoglobulins were effective serotype-specific opsonins for the bacteria (IgM requiring complement). High molecular weight PS is thought to contain the serotype determinant present in the 'O' side chain of LPS and recent analysis of the material by ^{13}C and proton nuclear magnetic resonance indicate that the 2 molecules have identical compositions (Pier, 1985). However, they differ in molecular size and immunogenicity.

1.5.2. Experimental studies.

Although the number of vaccines tested in human trials is fairly small as yet, and the trials themselves demonstrate varying degrees of success, a host of experimental vaccines have been developed over the last few years some of which show considerable potential for the future immunotherapy of *P. aeruginosa* in burns. With increased understanding of the problems of LPS-based vaccines in terms of toxicity and serotype specificity, researchers have concentrated on formulating less toxic vaccines, often employing alternative antigens of increased purity and common to all

serotypes, or have turned to improved passive immunotherapy regimes. The prospects in some cases look very bright.

High molecular weight polysaccharides (PS) have already been mentioned in terms of the immune response they elicit in human volunteers, but many experimental studies have also been conducted with such material. Pier (1982b) demonstrated that at high doses PS from *P. aeruginosa* Fisher immunotypes 1 and 2 gave cross protection in mice challenged with the heterologous immunotype and further showed that the 2 polysaccharides were cross immunogenic at these high doses but did not appear to be antigenically cross-reactive. Despite this, protection against challenge with IT-4 was not observed for either immunotype PS. In a burned mouse model, Pollack *et al* (1984) further demonstrated protection of mice by PS IT-1 or IT-2 against challenge with either immunotype although only antibodies versus the predominantly homologous immunotype were produced. The benefits of PS antigens is their lack of lipid A and core type sugars, KDO, heptose and glucosamine (Pier *et al*, 1983) which markedly decreases the side effects associated with immunization. Unfortunately, their immunogenicity compared to LPS is additionally reduced (Cryz *et al*, 1984a).

An alternative strategy to produce PS vaccines is to remove lipid A from purified LPS by mild acid hydrolysis. Furthermore, the immunogenicity of polysaccharide-based vaccines can be considerably augmented by conjugation with proteins. These conjugates induce helper T-lymphocytes which activate B lymphocytes against PS. An immunological memory is thus evoked which is not seen following immunization of PS alone (van de Wiel *et al*, 1987). Such an approach has been adopted by several researchers with encouraging results. Tsay and Collins (1984) generated a low molecular weight polysaccharide fraction which alone was non-immunogenic in mice. However, following oxidation, the PS was covalently coupled by reductive amination to derivatized bovine serum albumin and the resulting conjugate

shown to stimulate IgG antibody formation in mice. The antibodies so formed were reactive against LPS in an ELISA. The mice showed no signs of toxicity and were protected from IP challenge with *P. aeruginosa*. Moreover, serum from vaccinated mice protected burned and normal mice challenged with an otherwise lethal inocula of *P. aeruginosa*. The workers suggest that conjugation restored the immunogenicity of the PS similar to that of LPS without restoring endotoxicity. Van de Wiel *et al* (1987) recently reported coupling of an 'O' polysaccharide purified from Fisher immunotype 3 LPS to tetanus toxoid and also showed that the resulting conjugate stimulated high titres of IgG antibodies directed against LPS from that immunotype. At least a 1000-fold reduction of endotoxic activity was observed for this conjugate compared to homologous native LPS. The protective nature of the antibodies is currently being investigated.

A further improvement on the aforementioned technique is to couple the PS moiety to proteins derived from *P. aeruginosa*. The resulting conjugate thus elicits antibodies to 2 distinct antigens so, presumably, increasing the protective capacity of the vaccine. Cryz *et al*, (1986) made use of this idea by covalently binding PS derived from *P. aeruginosa* immunotype 5 LPS to purified exotoxin A. The resulting conjugate was composed of 27.5% PS (heterogeneous high molecular weight species) and 72.5% exotoxin A and was found to be non toxic as well as non-pyrogenic. IgG responses to both moieties were induced, those against exotoxin A being capable of neutralizing the cytotoxic effects of this compound. Furthermore, the antibodies were protective when evaluated in a murine burn wound sepsis model indicating that the conjugation technique, whilst successfully destroying toxicity of LPS and exotoxin A, allowed certain crucial epitopes on these molecules to be retained. The researchers have since demonstrated that the technique can be applied to 9 serotypes of *P. aeruginosa* with the same successful results and, that passive immunotherapy with serum raised

against the conjugates effectively prevented fatal *P. aeruginosa* burn wound sepsis (Cryz *et al*, 1987).

P. aeruginosa synthesizes several extracellular products outlined in section 1.3, many of which exert a deleterious effect on the host. Evidence that increased survival in patients correlated with high antitoxin titres (Cross *et al*, 1980; Pollack and Young, 1979) prompted researchers to investigate incorporating these toxins and enzymes, following inactivation, into vaccines for active immunization or raising antisera to the products for passive immunotherapy. As described above, toxoided exotoxin A coupled with PS material has been tested with promising results. Pavloskis *et al* (1981) evaluated the ability of formlin- and glutaraldehyde-treated exotoxin A plus adjuvant to protect burned mice from *P. aeruginosa* infection. The toxoid prepared from formalin treatment increased survival time and survival rate (50 to 85%) compared to controls (6 to 20%) and almost 100% survival was recorded when treatment was combined with a single dose of gentamicin within 24 h of infection. Less satisfactory results were achieved with the glutaraldehyde-treated toxin. Pavloskis *et al* (1977) and Snell *et al* (1978) found that passively transferred antitoxin afforded little protection against highly virulent *P. aeruginosa* strains, an observation later confirmed by Cryz *et al* (1983). The latter group reported that neither transfer of antitoxin or antielastase antibodies altered the course of infection or consistently decreased bacterial numbers. In contrast, these researchers demonstrated a high degree of serotype specific protection following transfer of anti LPS immunoglobulin. Toxoids of elastase, protease and toxin A have been incorporated into the multicomponent vaccine containing OEP (Homma, 1982a) as described in section 1.5.1. Experimental studies have also been conducted without exotoxin A. The vaccine gave significant protection against the same strain and serotype 5 but not against serotype 16 strains which appear not to

contain antigens cross-reacting with OEP (Okada *et al*, 1980). In the last year, Morihara and Homma (1986) reported development of a new method to prepare elastase toxoid using a chloroacetyl peptide derivative, with or without formalin and L-lysine pre-treatment. This procedure has been shown greatly to increase the antigenicity and immunogenicity of the final product whilst still destroying the enzyme activity. Antibody to the elastase toxoid had high enzyme neutralizing effects. Such a technique may help produce more efficaceous vaccines containing this enzyme.

Considerable controversy surrounds the potential of another group of *Pseudomonas* immunotherapeutic agents, namely the ribosomal vaccines. The original ribosomal fraction tested for its protective effect was described by Youmans and Youmans (1965). However, since then many investigators have implied that contaminating LPS is the effective immunogen in these preparations (Eisenstein, 1975; Hoops *et al*, 1976). Furthermore, Gonggrijp *et al* (1981) provided evidence that even though LPS was not detectable in the ribosomal fractions, antibodies to LPS were elicited following immunization with the vaccine. Nonetheless, *in vivo* mouse studies using a strain of mice non-responsive to LPS, demonstrated considerable protection by ribosomal vaccines from challenge with *P. aeruginosa* (Lieberman and Ayala, 1983). Certain evidence has also suggested that a cell surface protein may be an important factor in the ribosomal preparations which, it is thought, may be present on the ribosome as a nascent protein (Lieberman and Ayala, 1983). More recently, Lieberman *et al* (1986) tested ribosomal vaccines in a rat model where rats were subjected to a 20% total body surface burn. Administered prior to burning, the vaccines demonstrated 100% protection. Similar protection occurred if the vaccine was given post burn but 3 days prior to infection, although the value fell to 50% if the time interval before infection was reduced to 1 to 2 days. These results may possibly reawaken interest in the ribosomal vaccines.

With the realization that any LPS-based vaccine would require representative antigens from all serotypes to provide a full spectrum of protection and the associated problems this would entail in terms of production and possible toxicity, effort was made to select cellular antigens common to all serotypes and devoid of any toxicity. Obvious candidates were the outer membrane proteins which have been shown to be antigenically conserved in all 17 serotypes (Mutharia *et al*, 1982), and which have no endotoxic properties. Although certain doubts have been cast on the value of OMPs from members of the Enterobacteriaceae as vaccine candidates, such factors do not necessarily apply to non-enteric bacteria such as *P. aeruginosa* (Gilleland and Matthews Greer, 1987). The architecture of the cell envelope of non-enteric bacteria shows important differences from that of enteric rods, the latter having adapted to life in the gastrointestinal tract. Hence, LPS alone is found in the outer leaflet of the OM of these bacteria and they are resistant to the action of EDTA, whereas LPS and phospholipids may form the lipid outerleaflet of non-enteric bacteria and EDTA lyses cells of *P. aeruginosa* releasing LPS/protein complexes (Roberts *et al*, 1970). As a result, OMPs may be more accessible on the surface of the non-enteric bacteria and not shielded by PS from antibodies as seen in the Enterobacteriaceae (van de Ley *et al*, 1986). Several studies have demonstrated the recognition by host antibodies of *Pseudomonal* OMPs during the course of infection, both in experimental animal infections (Hedstrom *et al*, 1984) and human infection (Fernandes *et al*, 1981; Lam *et al*, 1983; Anwar *et al*, 1984). Gilleland *et al* (1984) were the first group to test an OMP vaccine in mice. They purified the porin protein F by column chromatography or by extraction from SDS-PAGE gels and showed that it protected mice from IP challenge with homologous and 2 heterologous strains of *P. aeruginosa*. Protection was lost following papain-treatment of the preparation demonstrating that protein and not

contaminating LPS were responsible for the protection observed. Protein F is a major OM antigen of all serotypes of *P. aeruginosa* and was shown to be expressed *in vivo* (Brown *et al*, 1984). Subsequently, Matthews Greer and Gilleland (1987) tested the protein in a more realistic model of burn wound infection and demonstrated significant protection above that provided by LPS against 6 heterologous serotype strains.

Another aspect of immunotherapy involving OMPs is the development of monoclonal antibodies (MCAs). Hancock *et al* (1985) isolated MCAs to *P. aeruginosa* protein F which resulted in passive protection against infection in 2 different mouse models. The MCAs were shown to opsonize *P. aeruginosa* viable cells for phagocytosis by PMNs. Sawada *et al* (1984) had previously tested the ability of MCAs to OMPs in preventing experimental infection with *P. aeruginosa*. Their results appeared less encouraging in that although they did offer protection it was not as effective as that seen with MCAs against LPS. However, this should not be seen as too disappointing since the possible advantage of cross-protection against all serotypes of the organism, including non-typable strains, achieved with antibodies to protein antigens may outweigh their reduced activity. The important question, then, is whether or not an effective concentration of MCAs to OMPs can be tolerated in humans without adverse effects (Gilleland and Matthews Greer, 1987). MCA technology is a promising area in terms of immunotherapy and has certain benefits especially in immunocompromised patients with rapidly developing infections. Other advantages include the ease of purification, high specificity and reproducibility. Unfortunately, certain drawbacks are associated with their use in humans, namely possible serum sickness and induced immunoregulatory defects, although some of these problems may be overcome with the development of human MCAs. Such MCAs have been isolated against the LPS of *P. aeruginosa* serotype 5, one of which was proven to have protective activity against peritoneal infection with *P. aeruginosa*

(Sawada *et al*, 1985). Human MCAs directed against target antigens may well have a place in the future immunotherapy of *P. aeruginosa* burn wound infection.

A further protein of *P. aeruginosa* present as a surface appendage is the flagella antigen which is known to be highly immunogenic (Mutharia *et al*, 1982; Anwar *et al*, 1984). Highly purified *P. aeruginosa* flagella preparations have been isolated and characterized (Montie *et al*, 1982a; Montie and Stover, 1983). Motility is thought to be an important virulence factor in burn wound sepsis (McManus *et al*, 1980; Craven and Montie, 1981) and antibodies which inhibit the functioning flagellum are likely to reduce the invasive capacity of the organism. The efficacy of flagella antigens as a vaccine has been tested in a burned mouse model (Holder *et al*, 1982). Protection was afforded against challenge with live *P. aeruginosa* in the burn area and was serotype independent but specific to H (flagella) serotype. There are only 2 major antigenic types of *P. aeruginosa* flagella (Ansorg, 1978) and Holder and Naglich (1986) have recently tested a divalent vaccine consisting of both flagella types. Mice immunized with the preparation were protected when burned and infected with *P. aeruginosa* and the protection was independent of both the flagella and somatic antigen of the challenge strain. No results were given concerning the protective capacity of this preparation if administered after the burn had been inflicted and before onset of infection, a sequence of events more probable in the clinical situation.

Passive immunotherapy with polyclonal antisera has been advocated as an alternative to active immunization in a number of the vaccine preparations discussed and may well find particular use in burn patients whose immunosuppressed condition may reduce the effect of direct vaccination. Until recently, administration of immune sera has been fraught with problems. Detrimental vasomotor reactions occurred following IV

injection of IgG (Barandun *et al*, 1964), so necessitating use of the IM route. The latter was associated with pain on injection, slow absorption and the inability to attain optimal blood levels of antibodies due to mass-volume considerations and, as a consequence, offered little promise. However, techniques have since been developed to modify IgG by reduction and alkylation such that it is safe for intravenous use (IGIV) (Schroeder *et al*, 1981 a and b) so raising considerable possibilities for this mode of therapy. IGIV is not limited by the volume that can be safely be deposited in the muscle. Indeed, doses of 100 to 1000 mg/kg body weight have been infused without major problems (Mease *et al*, 1981) and peak IgG levels in plasma are reached rapidly after infusion (Ochs *et al*, 1980). Holder and Naglich (1984) tested an IGIV preparation in burned mice. The immune globulin was shown to possess opsonizing antibody to all 7 Fisher immunotypes of *P. aeruginosa* (Pollack, 1983) and the results showed that protection did occur but that it was dose related. Large amounts of IGIV administered early in the infection process were required for full protection. Similar results were obtained by Pollack (1983). The reasons for this may include the fact that burned animals distribute and process passively transferred IgG differently from unburned animals. Furthermore, the IgG may be degraded by elastase produced by most clinical isolates of *P. aeruginosa* (Döring *et al*, 1981) so reducing its effect. Perhaps local administration of a protease inhibitor as described by Holder (1983) in conjunction with IGIV may help to circumvent this problem. Nonetheless, high doses of IGIV are well-tolerated and as such may prove a useful adjunct in immunotherapy. Additionally, Holder and Naglich (1984) demonstrated that adsorption of IGIV with heat-killed *P. aeruginosa* cells reduced its protective capacity. This was reduced even further by adsorption with formalin-killed cells implying that antibodies to components other than LPS, possibly heat-labile OMPs, flagella or pili may

be important in enhancing the protection observed. It is likely that IGIV prepared from individuals hyper-immune to *P. aeruginosa* by virtue of previous infection or immunization will offer even better protection. Indeed, Collins and Roby (1984) tested immune globulin prepared from human plasmas found to contain abnormally high levels of IgG to *P. aeruginosa* LPS. This preparation was several-fold more effective than normal human immune globulin in protecting burned mice from *P. aeruginosa* challenge.

Undoubtedly, therefore, great advances have been made in the last few years with regard to developing new, safe immunotherapeutic strategies to combat *P. aeruginosa* sepsis in burn patients. Researchers have explored a wide variety of approaches including both passive and active immunization, all aimed at improving existing human therapy. Undoubtedly, the future will see many of these ideas undergo the ultimate challenge, that of testing in human trials, the results of which could prove very exciting.

Regarding vaccination in general, major discoveries have been made in the past decade which will have far reaching effects in all areas of vaccine production including *P. aeruginosa* immunotherapy. Particular developments have occurred in the field of recombinant DNA technology as reviewed by Gilbert and Villa-Komaroff (1980). Such techniques, in theory, should overcome many of the current problems of purifying key antigens in sufficient quantity and in a suitable form and, may enable development of more highly purified subunit vaccines in which active components are enriched and non-essential, potentially harmful contaminants responsible for toxicity, are eliminated (Liew, 1985). The principles involved include identification of a key antigen which will stimulate high titres of protective antibodies, isolation of the genes coding for the antigen, insertion of the genes into a plasmid or other carrier, introduction of the complex into bacteria or other host cells and finally, expression of large amounts of the antigen required in a form readily purified. The realization

that even in such subunit vaccines, only a few of the sites determining antigenic specificity are important in provoking protective immunity whilst others may actually induce suppressor activity, has provided the impetus to develop synthetic peptide vaccines of even greater specificity. The latter would consist of only those peptides comprising the key epitopes free from unnecessary components. One problem associated with the potential use of these highly purified fragments is their lack of induction of effective cell-mediated immunity. For a similar reason, inactivated whole organisms are generally less effective than live vaccines. A possible solution may be to insert and transfect the genes of interest into attenuated heterologous carriers (Liew, 1985).

Most of these more recent aspects of vaccine technology are still in the developmental stages and, obviously, to be effective require identification of microbial antigens and epitopes which will stimulate protective immunity. It is crucial, therefore, to ascertain the antigens expressed by bacteria in *in vivo* infection, not simply those evident in cells cultivated in a conventional manner in the laboratory. The latter may not express all the determinants of virulence (Smith, 1977) seen in infection and key antigens and pathogenic factors which may be exploited in vaccine development may be overlooked. Moreover, those antigens which are expressed in *in vitro* cultures may differ in their physiological and chemical composition from their *in vitro* counterparts. Presently, researchers pay little attention to the conditions in which cells for vaccine production are cultivated. Perhaps if human trials of these components subsequently show less promising results than originally hoped for, this factor should be borne in mind. Studies of bacterial properties *in vivo* should significantly assist the future rational design of immunotherapeutic measures, whilst the technology to realize such measures is rapidly becoming available.

2. Materials

2.1. Bacteria.

Pseudomonas aeruginosa PAEW, PAGB PAJB and PASN were clinical isolates from burn patients, kindly supplied by Dr R. J. Wale (Burns unit, Birmingham Accident Hospital, Birmingham). The strains were 'O'-serotyped by Dr T. Pitt (Division of Hospital Infection, Public Health Laboratory Service, Colindale Ave, London) and found to be serotypes 0:4, 0:6, 0:11 and 0:6 respectively, as defined by the International Antigenic Typing Scheme (IATS).

P. aeruginosa strains of serotype 0:1, 0:3, 0:7, 0:8, 0:9, 0:10, 0:13 and 0:14 were clinical isolates kindly donated by Dr M. Noy (Microbiology Department, Selly Oak Hospital, Birmingham). Serotypes 0:2, 0:5, 0:15 and 0:17 were kindly supplied by Dr T. Pitt (Division of Hospital Infection, Public Health Laboratory Service, Colindale Avenue, London) and serotypes 0:12 and 0:16 were generous gifts of Dr W. Nichols (Bacteriology Department, John Radcliffe Hospital, Oxford). The laboratory strain *P. aeruginosa* PAO-1 (ATCC 15692) was also used in this study.

Clinical isolates of *Serratia marcescens* GH121, *Escherichia coli* GH126, *Klebsiella pneumoniae* GH320 and *Proteus mirabilis* GH110 were kindly donated by Dr S. Silverman (General Hospital, Birmingham).

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

2.2. Clinical material.

Serum and infected wound tissue from burn patients colonized with *P. aeruginosa* were supplied by Dr R. J. Wale (Burns unit, Birmingham Accident Hospital, Birmingham).

2.3. Chemicals.

Chemicals and reagents not specified in the text were supplied by BDH Chemicals Ltd. (Poole, Dorset), Sigma Chemical Company (Poole, Dorset) or Fisons (Loughborough, Leics.) and were of Analar grade or equivalent.

2.4. Preparation of glassware.

All glassware was fully immersed in Extran 5% v/v overnight at room temperature, rinsed once in distilled water , immersed in 1% v/v HCl, then rinsed six times in distilled water followed by three times in double-distilled water. The glassware was dried at 60°C, closed with aluminium foil and sterilized by dry heat at 160°C for 3 h.

2.5. Chemically defined media (CDM).

The composition of the chemically defined growth media for *P. aeruginosa* strains (CDM_{10}) is indicated in table 2.1. CDM_{10} was formulated by Noy (1982) for *P. aeruginosa* 6750. The concentration of each essential nutrient is sufficient to allow exponential growth to a theoretical optical density (OD) of 10. Addition of $FeSO_4.7H_20$ was omitted for iron-depleted-CDM (CDM-Fe). The constituents were dissolved in the appropriate volume of glass-double-distilled water and sterilized by autoclaving at 121°C for 15 min. Glucose and $FeSO_4.7H_20$ were autoclaved separately and aseptically added to the other sterile constituents.

2.6. Complex media.

Nutrient agar and Tryptone Soy broth (TSB) were obtained from Oxoid (London, S.E.1). Pseudomonas Isolation Agar was obtained from Difco Laboratories (Detroit, Michigan, U.S.A.). All were prepared according to the manufacturers instructions and sterilized by autoclaving at 121°C for 15 min.

Table 2.1. Composition of $CDM_{1.0}$

Nutrient	Final Concentration (mM)
NaCl	0.5
KCl	0.62
$K_2HPO_4.3H_2O$	3.2
$(NH_4)_2SO_4$	40.0
$MgSO_4.7H_2O$	0.4
Glucose	40.0
MOPS[1]	50.0
$FeSO_4.7H_2O$[2]	0.06

[1] MOPS (Morpholinopropane sulphonic acid), buffered to pH 7.8 with NaOH.

[2] $FeSO_4$ concentrate solution was acidified with concentrated H_2SO_4 to prevent precipitation when autoclaving.

The level of unbound iron in TSB was reduced by passing double-strength media through a column of Chelex-100 ion exchange resin (Bio-Rad, Watford, Herts). A glass gel filtration column (diameter 25mm) was filled with a slurry of resin in double-distilled water to a height of 200mm after settling. The column was prepared by washing in sequence with 1M HCl (2 bed volumes), double-distilled water (5 bed volumes), 1M NaOH (2 bed volumes), double-distilled water (5 bed volumes) and 0.7M sodium phosphate buffer pH 7.4 until the pH of the eluant was constant. The column was finally rinsed with 5 bed volumes of double-distilled water before passing through the media at a flow rate of 2ml/min/cm^{2}. The column was regenerated between the third and fourth media treatments using the sequence described. This procedure removes approximately 95% iron from TSB, and 99% of other metal cations (Kadurugamuwa *et al*, 1987). Treated broth would not support bacterial growth unless supplemented with magnesium. After treatment essential metal ions were replaced to a final concentration of: $MgSO_4$, $4x10^{-4}$M; $CaCl_2$, $5x10^{-7}$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 $(NH_4)_6Mo_7O_{24}$, $5x10^{-9}$M. The iron-depleted media was termed TSB-Fe. For iron-sufficient TSB $FeSO_4$ was added to chelex-treated media to a final concentration of 0.02mM.

2.7. Blood and serum.

2.7.1. Rabbit serum.

Antisera to *P. aeruginosa* whole cells cultivated in CDM-Fe was raised in rabbits (New Zealand White, female). Formaldehyde (37%) was added to the overnight culture to a final concentration of 4% and left at room temperature for 24 h. Killed bacteria were harvested by centrifugation at 5000 x g, washed twice in phosphate buffered saline (PBS) and resuspended to approximately 10^{10} cells/ml. A portion of the suspension was mixed with Freund's incomplete adjuvant (Difco Laboratories, Detroit, Michigan, U.S.A.)

to form an emulsion, and 1ml of the final preparation injected intramuscularly (IM), twice a week for the first 2 weeks. Immunization was subsequently carried out once a week for 4 weeks, then the rabbits rested for 1 month, followed by a booster immunization. Four days later the rabbits were bled from the marginal vein of the ear. The blood was allowed to clot for 2 h at room temperature then separated by centrifugation at 2000 x g for 10 min. The supernatant serum was removed and stored at -20°C.

Blood samples were additionally taken from rabbits in both peritoneal infection models (section 3.3) at regular time intervals during the course of the infection. Rabbits were bled from the marginal vein of the ear and serum obtained as described above.

2.7.2. Mouse serum.

A monospecific polyclonal antisera to individual *P. aeruginosa* OM proteins was raised in mice as described by Knudsen (1984). SDS-PAGE (section 3.4.3.) was used to separate OMPs using a 14%, single track polyacrylamide gel. Separated components were electrophoretically transferred to nitrocellulose (NC) paper (section 3.5.1) and narrow strips from both ends and the middle of the NC sheet were removed and stained with amido black 1% w/v to locate the position of the protein bands. NC strips bearing the required antigens were then excised from the corresponding unstained region of the blot and half the strips incubated in sodium periodate (0.5M, pH 4.0) for 1 h at 37°C followed by extensive washing in distilled water, in an attempt to reduce LPS contamination of the transferred proteins. Both untreated and treated strips were dried at 37°C, macerated and dissolved in a minimum volume of dimethyl sulphoxide (DMSO, approximately 500µl). Alhydrogel (PHLS, Porton Down, Wiltshire) was added dropwise to a final concentration of 12%v/v and the preparation mixed

vigorously to achieve maximum homogeneity. Alternatively, the DMSO-dissolved proteins were mixed with an equal volume of Freund's complete adjuvant (FCA) for the first immunization and Freund's incomplete adjuvant (FIA) for subsequent inoculations. Porton Laboratory mice (PHLS, Porton Down, Wiltshire) were inoculated subcutaneously with 200µl of the preparation at each of 3 sites on days 1, 6 and 9. Mice were bled on day 12 and serum separated from the blood as described (section 2.7.1.).

2.7.3. Human serum.

Blood samples were obtained by venipuncture from 6 volunteers (4 female, 2 male; age range 35 to 70) with no history of *P. aeruginosa* infection. Blood samples were similarly obtained from burn patients with *P. aeruginosa* wound sepsis, during the course of clinical investigation. The blood was allowed to clot for 2 h at 37°C, then centrifuged at 2000 x g for 10 min. The supernatant serum was collected and stored at -20°C.

2.7.4. Absorption of sera.

Lipopolysaccharide (LPS, 2mg) extracted from the appropriate *P. aeruginosa* serotype (section 3.2.4) was mixed with 1ml serum and incubated for 1 h at 37°C followed by 18 h at 4°C. Precipitated immune complexes were pelleted by centrifugation at 5000 x g for 15 min and the supernatant serum reabsorbed with a further 2mg LPS as described.

2.8. Equipment.

Automatic pipettes: Gilson pipetman, P-20, P-200 and P-1000 (Anachem Ltd., Luton, Beds.)

Balances: Sartorius 1702 balance (Sartorius Instruments Ltd., Belmont, Surrey.), Oertling HC22 (Oertling, Orpington, Kent).

Centrifuges: MSE high speed 18, MSE superspeed 50 (Measuring and Scientific Equipment Ltd., Crawley, Sussex), Beckman J2-21, Beckman LB-60M Ultracentrifuge (Beckman Instruments Inc., High Wycombe Bucks), and Eppendorf 5412 bench centrifuge (Baird and Tatlock Ltd., Atherstone, Leics.).

Flat bed apparatus: LKB 2117 Multiphor II electrophoresis unit (LKB-Produkter AB, Bromma, Sweden).

Fast Protein Liquid Chromatography System: High precision pump P-500, pH monitor, pH Flow-through electrode, V-7 injection valve, and Superose 12 HR 10/30 gel filtration column (Pharmacia, Uppsala, Sweden).

Freeze Dryer: Edwards Modylo freeze dryer (Edwards High Vacuum Ltd., Crawley, Sussex).

Gas Liquid Chromatography: Chromatography series 204 with PM8222 dual pen recorder (Philips/Pye-Unicam, Cambridge).

Gel electrophoresis apparatus: Mini-Protean system (Bio-Rad Laboratories Ltd., Watford, Herts.), or made in house (Aston Services).

Immunoblotting apparatus: 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 0.22μm (Gelman Sciences, Brackmills, Northampton).

Microscopes: Wild model M20, binocular, phase contrast microscope (Micro Instruments Ltd., Oxford), Hitachi 600 transmission electron microscope and Hitachi S450 scanning electron microscope (Hitachi, Japan)

pH meter: PTI-15 digital pH meter (Fisons Scientific Apparatus, Loughborough, Leics.).

Photography equipment: Nikon camera FG (Nippon Kogaku KK, Tokyo, Japan), with Kodak technical pan film 2415.

Power packs: Bio-Rad Model 500/200 (electrophoresis), Bio-Rad Model 250/2.5 (immunoblotting) (Bio-Rad Laboratories Ltd., Watford, Herts.).

Rotary evaporator: Buchi Rotorvapor-R (Fisons Scientific Apparatus, Loughborough, Leics.).

Sonicator: MSE Soniprep 150 (Measuring and Scientific Equipment Ltd., Crawley, Sussex).

Sonic bath: Bransonic B220 (Branson Cleaning Equipment Company, Connecticut, U.S.A.)

Spectrophotometers: Unicam SP600 UV spectrophotometer, Unicam SP8000 scanning UV spectrophotometer (Pye Unicam Instrumments Ltd., Cambridge), Cecil CE292 digital UV spectrophotometer (Cecil Instruments, Cambridge), LKB 2138 Uvicord S UV monitor with flow through cell and LKB 2210 chart recorder (LKB-Produkter AB, Bromma, Sweden).

Cuvettes for spectrophotometric measurements: plastic (Gallenkamp, Loughborough, Leics.) , quartz (Hellma, Westcliffe on Sea, Essex).

Syringes: Hamilton precision syringes 100µl, 50µl (Hamilton Bonaduz AG, Switzerland)

Whirlimixer: Fisons Scientific Apparatus (Loughborough, Leics.).

3. Experimental methods.

3.1. Growth Studies.

3.1.1. Measurement of bacterial cell concentration.

Spectrophotometric measurement of the optical density (OD) of a bacterial suspension was used to determine bacterial cell concentration. At low cell concentrations, a linear relationship exists between OD and cell concentration, as expressed by the Beer-Lambert law:

$$OD \alpha \frac{I_o}{I}$$

where I_o = incident light

I = emergent light

At higher cell concentrations this relationship does not hold due to secondary light scattering (Meynell and Meynell, 1970). Experiments have established that the Beer-Lambert law is obeyed up to an OD 0.3 (Kenward, 1975). Above this, OD values are less than anticipated for a given population of bacteria. Linearity can, however, be restored by dilution of the suspension to an absorbance less than 0.3 (but above ≈ 0.03). The wavelength of 470nm was chosen for measurements of OD. This was selected to minimize absorption by media constituents and bacterial metabolic products such as pyocyanin which has an absorbance maximum between 420 and 460nm. An OD of 1.0 at 470nm indicates a concentration of approximately 1×10^9 cells/ml (Anwar, 1981).

3.1.2. Growth measurements.

Growth of bacteria in complex media was measured by determining changes in OD with time. Inocula were prepared from an overnight culture grown in TSB, chelex-treated TSB (TSB-Fe) and chelex-treated TSB supplemented with 0.02mM $FeSO_4$ (TSB+Fe). Bacteria were harvested by centrifugation at 5000 x g for 10 min at room temperature and resuspended

to an OD_{470nm} of 1.0 in sterile saline. This suspension (0.25ml) was added to 24.75ml of the appropriate pre-warmed medium in a 100ml conical flask. The flasks were incubated at 37°C in a water bath and agitated with a shaking rate of 120rpm. Samples for measurement of OD were removed aseptically at designated intervals and were diluted where necessary. Undiluted samples were returned to the flask to prevent excessive reduction of culture volume, whist diluted samples were discarded.

3.2. Preparative techniques.

3.2.1. Preparation of bacterial outer membranes.

Outer membranes (OMs) were prepared by the method of Filip *et al* (1973). Cells were cultivated overnight at 37°C in an orbital shaking incubator and harvested by centrifugation at 5000 x g for 10 min at 4°C. The resulting pellet was washed once in 0.85% saline, then resuspended in 25ml distilled water. Cells were broken by 8 x 20-s pulses of sonication in an ice bath with 20-s intervals for cooling. Sarkosyl (N-lauryl sarcosine) was added to a final concentration of 2% v/v and the preparation incubated at room temperature for 1 h. Sarkosyl solubilizes the cytoplasmic membrane of *P. aeruginosa* but leaves the OM intact (Lambert and Booth, 1982). Any remaining unbroken cells were removed by centrifugation at 5000 x g for 10 min, and the supernatant then further centrifuged at 100,000 x g for 40 min at 4°C. The final pellet containing OMs was resuspended in 0.5ml distilled water and stored at -20°C.

3.2.2. Preparation of *P. aeruginosa* antigens for crossed immunoelectrophoresis.

Antigens were prepared by the method of Høiby and Axelsen (1973) with slight modifications as follows. An overnight culture of *P. aeruginosa* PAO-1 was used to inoculate 2 x 2L CDM-Fe and cells were cultivated to early stationary phase at 37°C in an orbital shaking incubator. Bacteria were harvested by centrifugation at 5000 x g for 10 min, washed once in 0.85% saline and re-centrifuged at 12000 x g for 10 min The pellet was weighed to determine the wet weight of cells and distilled water added to 3 times the wet weight. The cells were resuspended and broken by 3 times passage through a French Press followed by 6 x 20- s bursts of sonication in an ice bath with 20- s intervals for cooling. Remaining cell fragments were removed by centrifugation at 48,000 x g for 1 h at 4°C. The supernatant was

retained and divided into aliquots before storage at -20°C. Antigens were subsequently used for crossed immunoelectrophoresis (section 3.5.2). The protein content of the antigen preparation, determined by the Lowry assay (Lowry *et al*, 1951) was found to be 19 µg/ml.

3.2.3. Isolation of flagella.

Flagella preparations were isolated using a modification of the method described by Montie *et al* (1982a). Culture flasks (2x5L) each containing 2L TSB were inoculated from an overnight culture of bacteria and cultivated for 48 h at 37°C in a slowly rotating orbital incubator. Cells were harvested by centrifugation at 5000 x g for 10 min at 4°C and washed once in 0.85% saline. Flagella were sheared off by blending the suspension for 10 s in a commercial blender and whole cells were removed from the suspension by centrifugation at 5000 x g for 10 min. Centrifugation of the supernatant at 100,000 x g for 4 h at 4°C resulted in a crude flagella preparation which was further purified by repeated low and high speed centrifugations, until a clear pellet was obtained. The purity of the flagella was determined by SDS-PAGE (section 3.4.3) which resulted in a single band of approximate molecular weight 55K.

3.2.4. Extraction and purification of lipopolysaccharide (LPS).

Stationary phase bacteria cultivated in 4L TSB or TSB-Fe were harvested by centrifugation at 5000 x g for 10 min. The cells were washed once in 0.85% saline and resuspended in 40 ml double-distilled water. The suspension was transferred into a glass round-bottomed flask, previously dried to constant weight, and lyophilized. The flasks were reweighed to obtain the dry weight of the whole cells. LPS was extracted by the hot-phenol method of Westphal and Jann (1965) adapted as follows:

The cells were resuspended in 40ml Tris HCl 30mM pH 8.0 and broken by 10 x 20-s pulses of sonication in an ice bath, with 20-s intervals for cooling. Deoxyribonuclease (Bovine pancreas type III), ribonuclease (Bovine pancreas type 1-AS) and lysozyme were added to a final concentration of 0.1mg/ml and the preparation incubated for 2 h at 37°C. 10ml tetrasodium EDTA (0.5M) to remove cations binding LPS and protease (*Strep. griseus* type XIV; final concentration 1mg/ml) to digest protein were added and the mixture incubated overnight at 37°C with constant shaking. Protease was destroyed by heating to 70°C for 20 min. The digested cell suspension was mixed with an equal volume of 90% w/v aqueous phenol, preheated to the same temperature and stirred vigorously for 10 min. The mixture was centrifuged at 5000 x g for 30 min to permit phase separation, and the upper aqueous layer containing LPS was carefully removed. Care was taken not to disturb any remaining proteinaceous material at the interface. Two further extractions were carried out by reheating the phenol layer to 70°C and adding a further 50ml water at the same temperature. The pooled aqueous layers were dialysed against tap water for 48 h to remove phenol. The contents of the dialysis tubing were emptied into a flask and 50mM magnesium chloride added. LPS was pelleted by centrifugation at 100,000 x g for 4 h and the supernatant discarded. The sedimented gel of LPS was washed in double-distilled water, re-centrifuged, placed in a pre-weighed flask and lyophilized. The dried LPS was reweighed and absence of nucleic acids and protein in the purified product was confirmed by UV absorption at 260nm and 280nm using a Pye Unicam SP8000 scanning UV spectrophotometer.

3.2.5. Recovery and OM preparation of bacteria directly from infected burn wound tissue.

Samples of infected wound tissue, surgically removed from the patient during the course of treatment were placed in a sterile container immediately post-operation. Infecting organisms were identified by subculture onto selective media. The tissue was suspended in sterile, 0.85% saline and blended vigorously for 60 s in a commercial blender. Skin material and debris were removed by 2 to 3 processes of centrifugation at 1000 x g for 5 min and the pellet discarded. The supernatant was centrifuged at 5000 x g and the resulting supernant retained, lyophilized and used for immunoblotting (section 3.5.1). The pellet containing bacteria and remaining debris was washed 3 times in saline then resuspended in double distilled water. Bacterial cells were broken by 8 x 20-s pulses of sonication in an ice bath with 20- s intervals for cooling. Sarkosyl (N-lauroyl sarcosine) was added to a final concentration of 2% and the preparation incubated for 30 min at room temperature. Contaminating tissue debris and unbroken cells were pelleted by 2 centrifugation steps of 5000 x g for 10 min and the final supernatant centrifuged at 100,000 x g for 40 min. The OM pellet was washed twice and resuspended in double-distilled water (0.25ml) and stored at -20°C.

To determine the effect of the above procedure on OMP profiles of bacteria recovered from the tissue, a preliminary experiment was performed. *P. aeruginosa* PAEW was cultivated overnight in 500ml TSB-Fe, harvested by centrifugation, washed once in saline and divided into 2 portions. Bacterial OMs were prepared from one portion as described in section 3.2.1. and the protein profile of these OMs after separation by SDS-PAGE is shown in Fig. 3.1 (lane 2). The other portion of cells was added to a sample of burn wound tissue from a burn patient previously shown to have a very low level of contamination with Gram-negative organisms. The above procedure

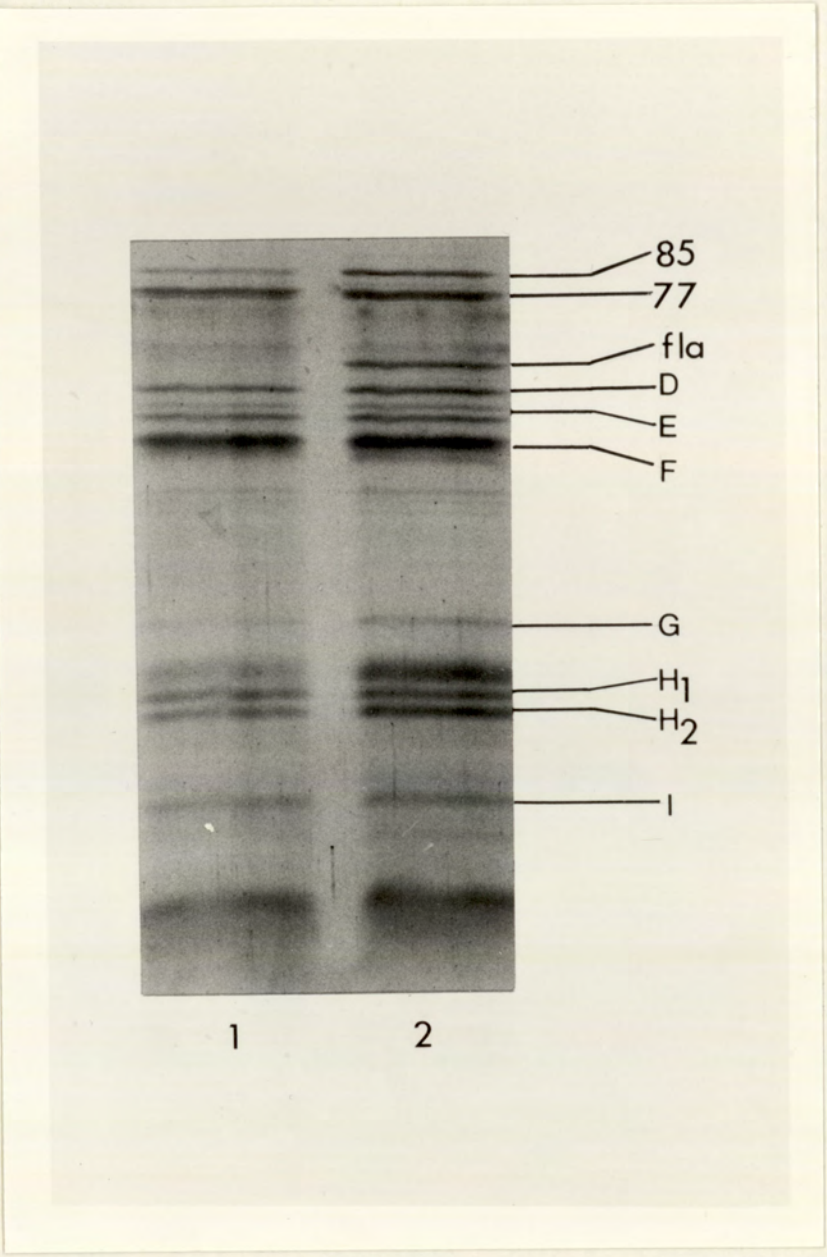


Fig. 3.1. SDS-PAGE of *P. aeruginosa* PAEW OMs prepared from whole cells as described in section 3.2.1. (lane 2) or prepared from cells extracted from wound tissue (lane 1). Letters refer to OM proteins labelled according to the scheme of Mizuno and Kageyama (1978) and numbers are molecular weights in kilodaltons. 'fla' represents flagella antigen.

was performed on this sample and the resulting OM pellet, gave an SDS-PAGE profile (lane 1) similar to that from untreated cells (lane 2). The only notable difference was the lack of the flagella antigen in cells extracted from wound tissue. This was most likely sheared off during blending.

3.2.6. Detergent extraction of *P. aeruginosa* OMs.

OM pellets prepared from *P. aeruginosa* cells cultivated in CDM-Fe were extracted with a range of detergents to determine those proteins which were soluble under the stated conditions. The pellets were suspended in the detergent solution for 1 h at room temperature, then centrifuged at 100,000 x g for 40 min. The supernatant was removed and the pellet resuspended in distilled water to the same volume. Both soluble and insoluble fractions were analyzed by SDS-PAGE. The detergents selected included 1 and 5% Tween 20, 1 and 5% Tween 80, 1% Triton X 100, 1% Nonidet P 40, 1% n-octyl β-D-glucoside, 0.3% Empigen BB (Allbright and Wilson, Whitehaven, Cumbria), 1% CHAPS, 1% cetyl trimethyl ammonium bromide (CTAB), 1% SDS and 1.5% sodium deoxycholate, in 50mM Tris HCl pH 8.0. Detergent solutions + 10mM EDTA were additionally tested.

3.2.7. Preparation of samples for transmission electron microscopy.

Silastic discs removed from the peritoneum of rabbits with biomaterial-associated infection (section 3.3.2) were exposed to specific antibody to partially stabilize the biofilm glycocalyx (Costerton, 1979), before being processed for transmission electron microscopy (TEM). This involved incubating the discs in undiluted antisera for 1 h. The antisera was raised in rabbits against formalin-killed whole cells of *P. aeruginosa* PAO-1 (section 2.7.1) and was heated to 56°C for 20 min to destroy complement. Following incubation the disc surfaces were washed in phosphate-buffered saline and fixed for 2 h in 5% glutaraldehyde (Ladd

Research Industries Inc. U.S.A.) in cacodylate buffer (0.1M cacodylic acid, pH 7.2) containing 0.15% ruthenium red (Aldrich Chemical Co., Milwaukee, U.S.A.). The biofilm was then scraped from the surface of the discs using a scalpel blade and enrobed in 4% agar (Bacto-Agar, Difco Laboratories, Detroit, U.S.A.). The enrobed material was washed 5 times in cacodylate buffer containing 0.05% ruthenium red (10 min per wash) to remove residual glutaraldehyde. Post-fixation in 2% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, U.S.A.) in cacodylate buffer (0.05% ruthenium red) for 2 h was followed by 5 further washes in buffer. Dehydration was performed with graded concentrations (0 to 100%) of acetone (20 min in each) and subsequently with 100% propylene oxide (Fisher Scientific Co. New Jersey, U.S.A.), for 10 min. Embedding resin was made with a Spurr low-viscosity kit (Electron Microscopy Sciences, Fort Washington, U.S.A.) as described by Spurr (1969). A 3:1 mixture of propylene oxide and resin was prepared and specimens transferred to a series of propylene oxide-resin mixtures until final immersion in pure resin. The resin was polymerized at 60°C for 8 h and sections cut on a LKB Ultratome III (LKB-Produkter AB, Sweden). The sections were mounted on 200 mesh 3mm copper grids and stained with 1% uranyl acetate pH 5.0 (Fisher Scientific Co., New Jersey, U.S.A.) and lead citrate (Eastman Kodac Co., New York, U.S.A.) as described by Reynolds (1963). They were subsequently coated with evaporated carbon using a Balzer BA 360 Freeze-etching device and examined with a Hitachi 600 transmission electron microscope (Hitachi, Japan) at an acceleration voltage of 60kV, by Joyce Nelligan (Department of Biology, University of Calgary, Canada).

3.2.8. Preparation of samples for scanning electron microscopy.

Infected discs removed from the rabbit peritoneum were placed for 2 h at room temperature in 0.1M cacodylate buffer (pH 7.2) containing 5%

glutaraldehyde and 0.05% ruthenium red. The samples were washed 5 times in cacodylate buffer and dehydrated with graded concentrations of ethanol (20 to 100%) and Freon 113-ethanol solutions (30 to 100%). Following air drying samples were sputter-coated with gold and examined using a Hitachi S450 scanning electron microscope (Hitachi, Japan) by Ushi Sabharwal (Department of Biology, University of Calgary, Canada).

3.3. Animal models of infection.

The aim was to establish 2 models of peritoneal infection in rabbits: a) a disseminated peritonitis in which bacteria spread to colonize tissue surfaces throughout the peritoneum, and b) a localized infection characterized by bacteria adherent on the surface of an implanted biomaterial (Silastic sheeting; Dow Corning Corporation, Medical Products, Midland, Michigan, U.S.A.), used in Tenckhoff catheters (Tenckhoff and Schechter, 1968). The latter was termed biomaterial-associated infection.

3.3.1. Disseminated infection model.

To achieve a disseminated peritonitis it was necessary to provide a focus of infection. One sterile disc of Silastic sheeting (diameter 1.5cm) was placed in 25ml CDM-Fe and the flask inoculated with *P. aeruginosa* PAO-1. Following overnight growth at 37°C, the disc was removed from the flask and surgically implanted, unwashed, into the rabbit peritoneum (New Zealand white, female). A post mortem was performed on any rabbits which died and samples of pus or peritoneal fluid were recovered, lyophilized and subsequently used in immunoblotting studies (section 3.5.1).

3.3.2. Biomaterial-associated infection model.

A device as shown in Fig. 3.2 was used to establish a biofilm of bacteria on the surface of Silastic sheeting. The device consisted of a central plastic rod (length 1.5 cm) which acted as a support for 10 discs of Silastic sheeting (diameter 1.5 cm), sandwiched between hard plastic discs. Each device was sterilized with ethylene oxide for 4 h. *P. aeruginosa* PAO-1 was cultivated overnight in CDM-Fe, harvested by centrifugation at 5000 x g for 10 min and washed in sterile phosphate buffered saline (PBS). Washed cells were resuspended in sterile PBS to the order of 10^5 organisms/ml, and devices incubated in the dilute bacterial suspension in a

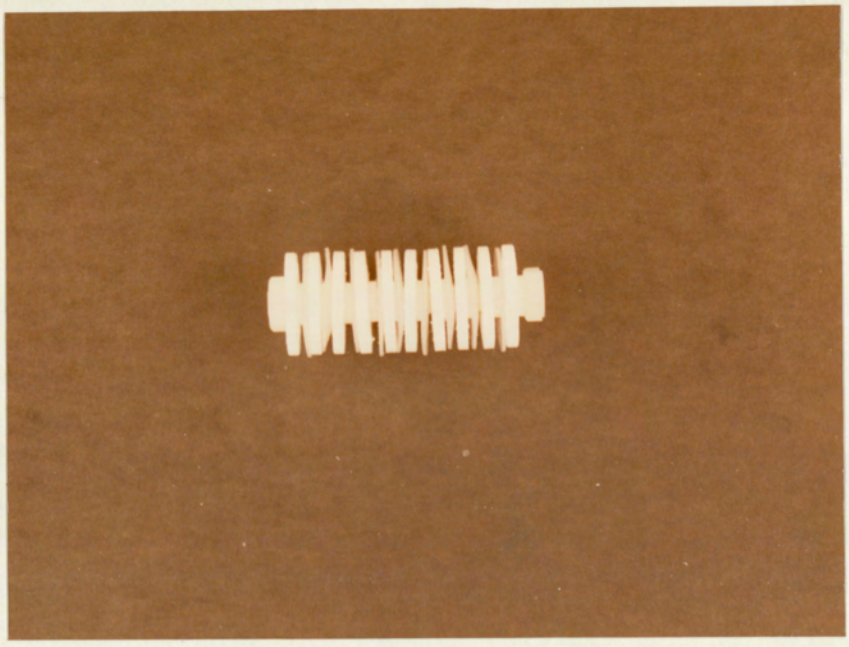


Fig. 3.2. Device implanted into rabbits in the biomaterial-associated infection model, consisting of a central rod supporting 10 discs of Silastic sheeting sandwiched between white plastic discs.

sterile plastic container on a rocking platform for 2 h at 37°C. Two such devices were then surgically implanted into the peritoneum of each rabbit.

A laparotomy was performed on rabbits in both models of infection, 4 days and 4 weeks after initial surgery to investigate the spread of infection throughout the peritoneum (ie evidence for dissemination). Animal surgery was carried out by Dr M. Olson and C. Barlow (Department of Biology, University of Calgary, Canada)

3.3.3. Viable counts of bacteria attached to biofilm devices/discs.

Replicate biofilm devices following incubation in the dilute bacterial suspension, or discs after overnight incubation in bacterial culture, were placed in 20ml sterile PBS and the devices dismantled under aseptic conditions. The surfaces of all discs were scraped with a sterile scalpel blade and the scraped material dispersed by vortexing for 60 s and by sonic bath disruption for 60 s (Ladd *et al*, 1985). The latter step serves to break up bacterial clumps. The amplitude is very low and does not affect the viability of the cells. Serial dilutions in sterile PBS were performed and 0.1ml aliquots were spread-plated onto nutrient agar to quantify the number of viable bacteria associated with the discs before implantation into the rabbit peritoneum. The same procedure was performed on devices/discs removed from the peritoneum 4 weeks after implantation. In this instance, material scraped from the discs was subjected to 2 x 30-s periods of blending in a commercial blender in addition to vortexing and sonic bath disruption, to aid dispersal of bacteria.

3.4. Analytical techniques.

3.4.1. Fatty acid analysis.

Fatty acids ester-linked to cellular phospholipids were extracted from whole cells by alkaline hydrolysis. However, fatty acids present in extracted LPS are amide-linked to the diglucosamine molecules in lipid A and required the harsher acid hydrolysis to release them. Free fatty acids were subsequently analysed by gas liquid chromatography (GLC).

3.4.1.1. Alkaline hydrolysis of whole cells.

Alkaline hydrolysis was carried out according to the method of Moss (1978). Whole cells of *P. aeruginosa* cultivated overnight in TSB or TSB-Fe were harvested by centrifugation at 5000 x g for 10 min washed once in saline and resuspended in saline to OD_{470nm} 5.0. The cell suspension (0.5ml) was placed in sealable tubes with Teflon-lined caps (Sterilin, Teddington, Middlesex), previously cleaned by soaking overnight in Extran 5% v/v followed by 1M H_2SO_4. The cells were mixed with 5% NaOH / 50% aqueous methanol (2.5ml) and the tubes sealed and heated at 100°C for 30 min. On cooling, the contents were adjusted to pH 2.0 with concentrated HCl. Methyl esters of the free fatty acids were formed by the addition of 2.5ml boron trifluoride-methanol complex (14% w/v boron trifluoride) followed by heating to 80°C for 5 min. Fatty acid methyl esters were extracted from the preparation with 5ml chloroform/hexane (1:4). The upper solvent layer was removed and evaporated to dryness under vacuum in a rotary evaporator. The residue was redissolved in 40µl hexane and evaporated at room temperature to a final volume of approximately 10µl.

3.4.1.2. Acid hydrolysis of whole cells or LPS.

Extracted LPS (10mg) or whole cells as described above were placed in sealable tubes and hydrolyzed by boiling in 6M HCl for 4 h. The tube contents were allowed to cool and methyl esters formed by the addition of 2.5ml boron trifluoride-methanol complex (14% w/v). Fatty acid methyl esters were extracted as for alkaline hydrolysis.

3.4.1.3. Gas liquid chromatography.

Extracted fatty acid methyl esters (2µl) were loaded onto the GLC column packed with 3% SP-2100 DOH on 100/120 Supelcort (Supelco Chromatography Supplies, Supelchem, Sawbridgeworth, Herts). The column was operated under the following conditions:

Column temperature: 150 to 225°C at 2°C/min increases.

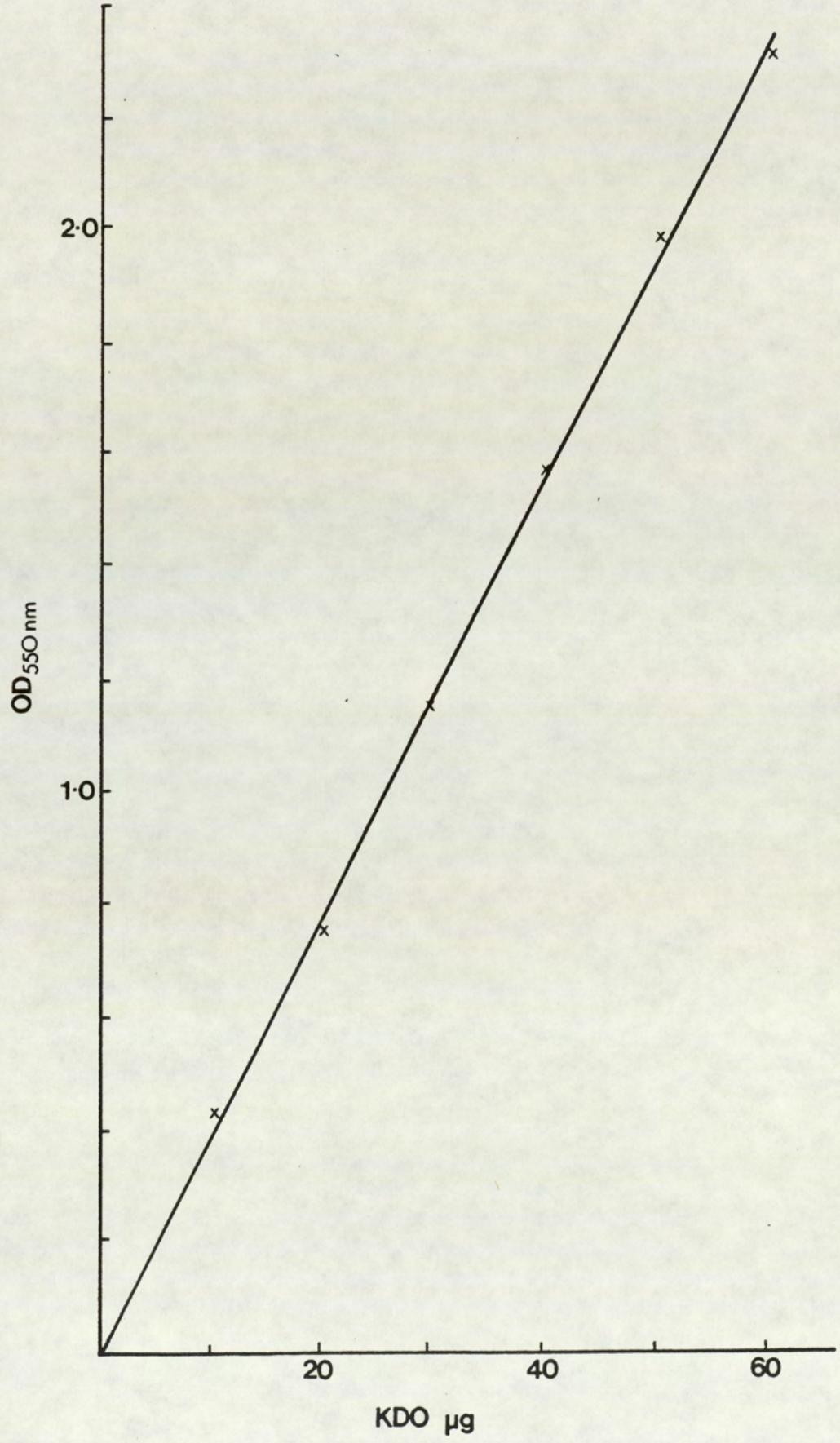
Gas pressures	:	Hydrogen	14.5psi
		Air	6.5psi
		Nitrogen	15.0psi

Fatty acids were identified by retention time comparisons to a standard mix of bacterial acid methyl esters (Supelco Chromatography Supplies, Supelchem, Sawbridgeworth, Herts.). Integration readings were calculated for each peak and expressed as a percentage of the integration reading obtained for all the peaks in the sample.

3.4.2. 2-keto 3-deoxy-D-manno-2-octulosonic acid (KDO) assay.

The method used was based on that of Osborn (1963). Samples of KDO standard (10 to 100µg) or 50µl extracted LPS (10mg/ml) were made up to 0.25ml with 0.05M H_2SO_4 and hydrolyzed by heating in sealed tubes at 100°C for 30 min. On cooling, 0.25ml periodic acid (0.025M in 0.0625M H_2SO_4) was added and the samples warmed to 55°C for 20 min. This was followed by addition of 0.5ml sodium arsenite (2%w/v in 0.5M HCl) and thorough mixing.

Fig 3.3. Calibration curve for the estimation of 2-keto 3-deoxy-D-manno-2-octulosonic acid (KDO).



Thiobarbituric acid (0.3% w/v, 2.0ml) was added and the tubes sealed and heated to 100°C for 20 min. On cooling, the absorbance at 550nm was measured. If signs of precipitation were apparent, the samples were extracted with 5ml concentrated HCl + n-butanol (5 + 95% v/v) followed by centrifugation at 5000 x g for 10 min and removal of the upper layer for spectrophotometric measurement. Standards and samples were assayed in duplicate. A standard calibration curve of KDO against OD_{550nm} was plotted (Fig. 3.3) to enable the KDO content of LPS samples to be determined.

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

OMPs were separated by gel electrophoresis using the method of Lugtenberg *et al* (1975), modified by Anwar *et al* (1983c). Slab gels were prepared, consisting of a running gel and a stacking gel. The composition of each is outlined in table 3.1.

Solutions 1 to 5 of the running gel were mixed in a glass beaker and polymerization initiated by the addition of NNN'N'-tetramethylethylene diamine (TEMED). The gel mixture was poured between glass plates separated by 1mm plastic spacers and allowed to set for 10 min. The stacking gel was prepared in a similar manner, poured on top of the running gel and a teflon comb inserted between the plates. The gel was polymerized fully then the comb removed, so creating wells for sample application. Samples were denatured by heating at 100°C for 10 min with an equal volume of sample buffer (table 3.1) before loading onto the gel. Both electrode buffers contained 0.025M Tris, 0.19M glycine and 0.1% SDS. A constant current of 40mA was applied to the gel and electrophoresis continued until the tracking dye had moved 12cm.

Gels were either used directly for immunoblotting (section 3.5.1) or stained for protein overnight in a solution of 0.1% Coomassie brilliant

Table 3.1 . Composition of running gel, stacking gel and sample buffer for SDS-PAGE.

Ingredient	Running Gel		Stacking Gel	Sample buffer
	12%	14%		
Stock 1	15.75ml	18.75ml		
Stock 2			5.0ml	-
10% w/v SDS	1.5ml	1.5ml	0.3ml	5.0ml
1.5M Tris[1] pH 8.8	18.75ml	18.75ml	-	-
0.5M Tris pH 6.8	-	-	7.5ml	2.5ml
Distilled water	21.5ml	18.5ml	16.0ml	5.0ml
TEMED[2]	0.14ml	0.14ml	0.8ml	-
10% w/v APS[3]	0.2ml	0.2ml	0.1ml	-
2-mercapto-ethanol	-	-	-	0.3ml
5% bromo-phenol blue	-	-	-	0.2ml
Glycerol	-	-	-	2.5ml

Stock 1 = 44% w/v acrylamide and 0.8% w/v N,N'-methylene-bis acrylamide (Bis)

Stock 2 = 30% w/v acrylamide and 0.8% w/v Bis

[1] Tris (hydroxymethyl) amino ethane

[2] NNNN' Tetramethylethylene diamine

[3] Ammonium persulphate (freshly prepared)

blue R-250 in 50% methanol-10% acetic acid. They were subsequently destained in 5% methanol-10% acetic acid and photographed using diffuse transmitted light.

Molecular weights of OMPs separated by SDS-PAGE were estimated by comparison with standard marker proteins. The standards used were phosphorylase A (97.4K), bovine albumin (66K), egg albumin (45K), glyceraldehyde -3 phosphate dehydrogenase (36K), carbonic anhydrase (29K), trypsinogen (24K), trypsin inhibitor (20.1K) and α-lactalbumin (14.2K).

Later work was performed with the Mini Protean system (Bio-Rad Laboratories Ltd; Watford, Herts.) using 0.25 mm plastic spacers. This system was operated at a constant voltage of 200V, and offered the advantage of faster separation times without loss of resolution. In addition, it enabled analysis of small sample quantities and a more economical use of gel solutions.

3.4.4. Characterization of LPS.

Purified LPS was separated by SDS-PAGE using 14% acrylamide gels and the buffer system described for gel electrophoresis of OMPs (section 3.4.3). For immunoblotting (section 3.5.1) LPS was separated on 12% acrylamide gels to improve resolution of high molecular weight material.

Alternatively, proteinase-K digests of bacterial OM preparations were used, based on the method of Hitchcock and Brown (1983). OMs (100µl) were denatured at 100°C for 10 min with 60µl sample buffer (table 3.1). On cooling, 40µl of sample buffer containing 2.5mg/ml proteinase-K was added and the preparation warmed to 60°C for 1 h. Samples were loaded onto polyacrylamide gels as for purified LPS.

In both systems electrophoresis was carried out at a constant current of 25mA until the dye front had travelled 12 cm. Gels were either used

directly for immunoblotting (section 3.5.1), or stained with silver (section 3.4.5)

3.4.5. Silver stain of LPS.

LPS fractionated by SDS-PAGE was stained by the method of Tsai and Frasch (1982). The gel was immersed overnight in 40% ethanol- 5% acetic acid solution. This was replaced with fresh solution containing 1% periodic acid and the LPS oxidised for 1 h. After 3 x 30 min washes with double-distilled water to remove unreacted periodic acid, staining reagent was poured over the gel. The staining reagent was freshly prepared by slowly adding 5ml of 20% silver nitrate solution to a mixture of 2ml concentrated ammonium hydroxide and 28ml 0.1M NaOH. The solution was made up to 150ml with double-distilled water, and the gel agitated in the reagent for 45 min. Further washing was performed as described and the water replaced with formaldehyde developer containing 50mg citric acid and 0.5ml formaldehyde (37%) per litre. When the LPS in the gel had stained to the desired intensity (5 to 20 min), colour development was terminated by replacing the developer with 40% ethanol- 5% acetic acid. Gels were photographed immediately. For gels run on the Mini Protean system, staining, washing and developing times were reduced to 30 min each.

3.4.6. Gel filtration chromatography.

Gel filtration chromatography was performed in an attempt to purify *P. aeruginosa* IRMPs. The method of separation is based on the flow properties of a protein mixture through a bed of cross-linked polymer.

A Superose 12 HR 10/30 chromatography column was selected (column length 30cm, diameter 1.5cm, Pharmacia, Uppsala, Sweden) composed of cross-linked agarose, with optimal separation in the molecular weight range $1x10^3$ to $3x10^5$.

All buffers and samples were filtered through 0.22μm cellulose acetate filters (Acrodiscs; Gelman Sciences, Brackmills, Northampton) and degassed under vacuum before use. The column was equilibrated with 2 volume changes of buffer before application of samples which were loaded on to the column by loop injection. Buffer was pumped through the column at a rate of 25ml/h using the Pharmacia FPLC system with a high precision pump (model P-500). Absorbance of the eluant was monitored at 280nm and fractions collected and concentrated by lyophilization.

Two buffer systems were used during the study:

a) 50mM Tris/HCl pH 8.0, containing 0.1M NaCl, 0.02% sodium azide, 1mM phenylmethylsulphonyl fluoride (PMSF) and either 1% or 0.1% SDS.

b) 0.1M glycine/NaOH pH 9.5, containing 5mM EDTA, 0.02% sodium azide, 1mM PMSF and 1.5% sodium deoxycholate.

3.5. Immunological techniques.

3.5.1. Immunoblotting

The transfer of OM antigens, separated by SDS-PAGE to a solid phase for immunoreaction was performed by a modification of the method of Towbin *et al* (1979). Nitrocellulose (NC) paper (Transblot membrane, pore size 0.45μm, Bio-Rad Laboratories Ltd, Watford, Herts.) was soaked in transfer buffer (25mM Tris, 193mM glycine and 20% methanol, pH 8.3) and laid on a sheet of chromatography paper (Whatman Ltd., Maidstone, Kent). Following electrophoresis, the gel was placed on top of the NC paper and overlayed with a second sheet of chromatography paper. The NC paper, gel and chromatography paper were in turn sandwiched between 2 Scotchbrite pads (Bio-Rad Laboratories Ltd., Watford, Herts.) in a Transblot cassette. The cassette was placed in a Transblot cell (Bio-Rad Laboratories Ltd., Watford, Herts.) filled with transfer buffer, previously cooled to 4°C, and the whole apparatus was surrounded by ice. Electrophoretic transfer of proteins was carried out at 80V for 1 h then overnight at 50V and qualitative transfer of proteins was confirmed by staining a duplicate piece of NC paper directly with 1% w/v amido black in 55% methanol- 10% acetic acid. Efficiency of protein transfer is known to be a function of molecular weight (Burnette, 1981; Vaessen *et al*, 1981) and these conditions were necessary to ensure complete transfer of high molecular weight proteins. LPS fractionated by SDS-PAGE was transferred to NC paper at 30V overnight.

Following transfer the NC sheet was soaked in Tris buffered saline (TBS; 10mM Tris HCl, 0.9% w/v NaCl, pH 7.4) containing 0.075% v/v Tween 20 (TBS/Tween) for 1 h to saturate non-specific binding sites in the nitrocellulose (Batteiger *et al*, 1982) and possibly to promote renaturation of proteins (Towbin and Gordon, 1984). The paper was then incubated for 4 h at 37°C in rabbit, mouse or patient serum, diluted 1 in 20 in TBS/Tween, unless otherwise stated. After washing 3 times with TBS the immunoblots

were incubated for a further 2 h at 37°C in horseradish peroxidase-conjugated goat anti-human IgG, IgM or IgA (Miles Scientific, Rehovot, Israel) or rabbit anti-mouse IgG or IgM, diluted 1 in 2000 in TBS/Tween. For immunoblotting with rabbit serum, peroxidase-conjugated protein-A (25µg / 100ml TBS/Tween) was used. Protein A binds to the Fc portion of numerous mammalian IgG immunoglobulins (Goding, 1978) hence its use in a variety of immunological techniques. Finally, after further washing, antigenic sites were visualized with a 25µg/ml solution of 4-chloronaphthol in TBS containing 0.01% hydrogen peroxide.

Strip-blotting was performed following separation of OM antigens by SDS-PAGE on a single track 14% acrylamide gel and electrophoretic transfer as described. The NC sheet was then cut into 5mm strips before incubating in serum. This technique enabled strips bearing replicate patterns of antigens to be probed with a range of sera.

3.5.2. Crossed immunoelectrophoresis.

Crossed immunoelectrophoresis was performed according to the procedures of Høiby and Axelsen (1973) and Kroll (1973), with slight modifications as follows:

Tris-barbital buffer, containing 0.073M Tris, 0.024M barbituric acid C1V (Fisher Scientific Co. New Jersey, U.S.A.) and 0.35mM calcium lactate, pH 8.6, was used to dissolve 1% w/v agarose of medium electroendosmosis, 0.16 to 0.19 (Seakem, Marine Colloid, FMC Bioproducts, Rockland, U.S.A.). After boiling, the agarose gel was cast onto a glass plate (8x12cm) in an agarose/surface area ratio of 0.18 ml/cm^2. On setting, a number 2 cork borer was used to punch wells in the gel in a line at 1cm intervals. Human serum (2µl) containing bromophenol blue (10mg/ml) was placed in the top well and *P. aeruginosa* antigen (6µl; section 3.2.2.) loaded into the remaining wells. The gel was placed on a water-cooled flat-bed

immunoelectrophoresis apparatus (LKB-Produkter AB, Bromma, Sweden). Paper wicks (Whatman No. 4 Chromatography paper, Fisher Scientific Co., New Jersey, U.S.A.) soaked in Tris-barbital buffer were used to make the buffer-gel connections. Electrophoresis was carried out at a constant voltage of 10V/cm until the serum albumin marker had migrated 26mm. The agarose gel was cut into strips (1cm x 6cm) containing the separated antigens. Individual strips were transferred to the hydrophilic side of Gel Bond support film (6 x 5cm; FMC Bioproducts, Rockland, U.S.A.). A brass bar was placed at a distance of 1cm from the first dimension strip and an intermediate gel (1% w/v agarose in Tris-barbital buffer containing 0.145M NaCl) cast in the intervening space. Agarose (1% w/v) in Tris-barbital buffer was cooled to 50°C and rabbit serum incorporated (120µl serum/ml). The brass bar was removed and after thorough mixing, the serum-containing gel was cast onto the remaining Gel Bond adjacent to the intermediate gel. Electrophoresis in the second dimension (perpendicular to the first) was carried out at 3V/cm for 18 h.

Following electrophoresis, non-precipitated proteins were removed by pressing and washing the gel casts (Weeke, 1973). The gels were placed on a flat surface, covered with 6 sheets of blotting paper (Whatman No. 1 Chromatography paper, Fisher Scientific Co., New Jersey, U.S.A.) and a glass plate plus weights and pressed evenly for 10 min to reduce the gel to a thin film. They were then soaked in 0.85% NaCl for 20 min, pressed for 10 min and washed for 20 min in double distilled water. After pressing for a further 10 min, the gels were dried with a hair-drier and stained for 4 min with 0.5% w/v Coomassie brilliant blue R-250 in 40% ethanol-10% acetic acid. Destaining was carried out for 5 min in 40% ethanol-10% acetic acid.

Crossed-line immunoelectrophoresis was performed by incorporating antigen (LPS extracted from *P. aeruginosa* PAO-1) into the intermediate gel.

4. Effect of iron content of culture media on growth and OM antigens of *P. aeruginosa*.

4.1. Growth of *P. aeruginosa* under iron-sufficient and iron-depleted conditions.

Chelex-100 ion exchange resin was used to reduce the level of iron in tryptone soy broth (TSB), as described by Kadurugamuwa *et al* (1987). This procedure has been shown to remove 95% of iron from TSB and 78% from NB. Metal cations other than iron are also removed, hence prior to use, these were replaced to the concentrations present in untreated media.

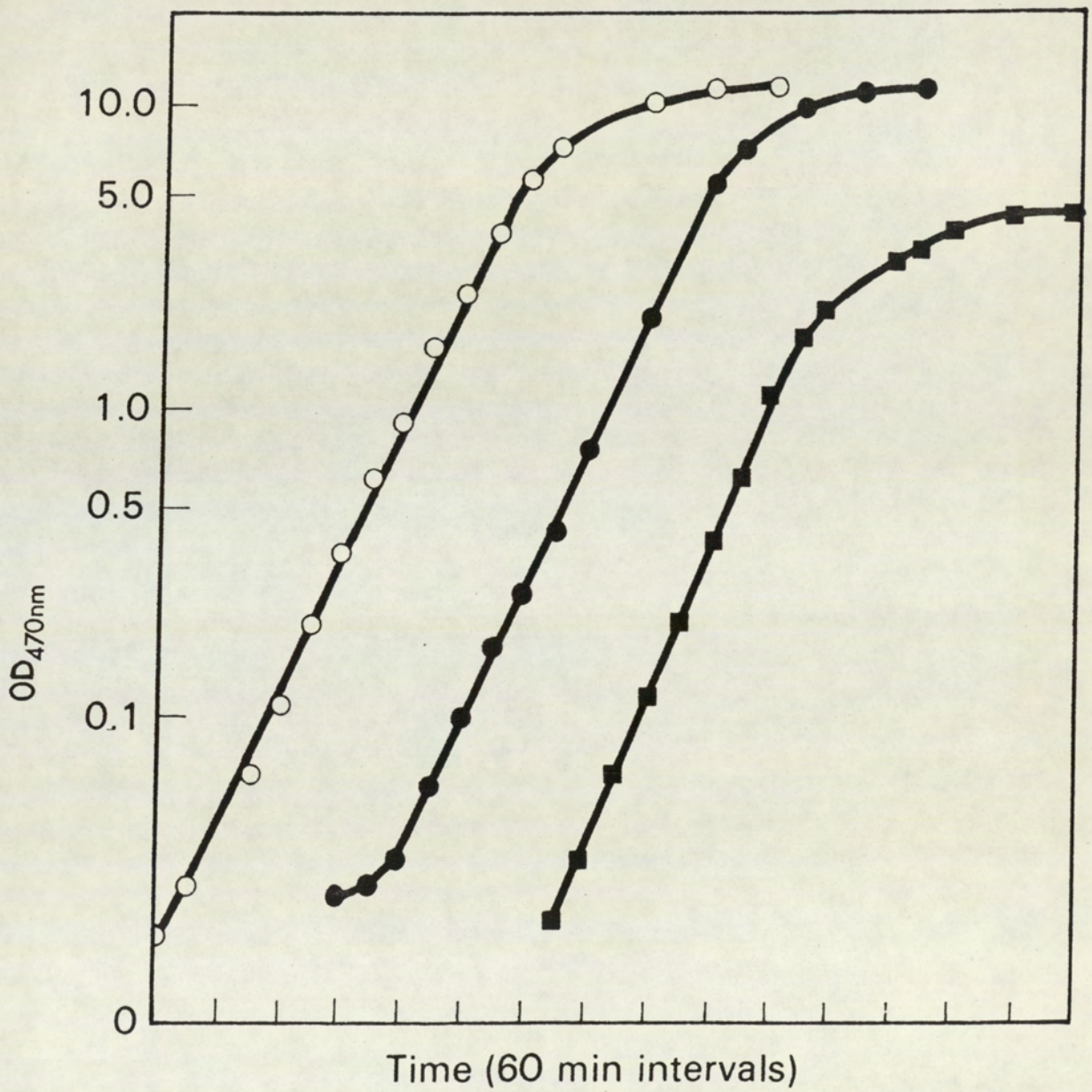
The effect of iron-depletion on the growth of *P. aeruginosa* PAEW was determined by following optical density (OD) measurements of bacteria cultured in TSB, Chelex-treated TSB (TSB-Fe) and Chelex-treated TSB supplemented with 0.02mM $FeSO_4$ (TSB+Fe). The resulting growth curves are shown in Fig. 4.1.

For growth in TSB or TSB+Fe the doubling time was 42 min and the growth rate 0.48 h^{-1}. The onset of stationary phase occurred at an OD_{470nm} of 5.8 in both instances.

Growth in TSB-Fe from an overnight inoculum in TSB-Fe resulted in the same doubling time of 42 min. However, the onset of stationary phase occurred at an OD_{470nm} of 2.4 and the overall bacterial yield was reduced.

In iron-sufficient conditions, if all essential nutrients are present in excess the cells stop growing exponentially either when secondary metabolites in the culture medium reach toxic levels, or as a result of inadequate oxygenation. In iron-depleted media, iron, essential for many metabolic processes, becomes the limiting nutrient before secondary metabolites accumulate sufficiently to prevent growth. Hence the final bacterial population is reduced.

Fig. 4.1. Growth of *P. aeruginosa* in TSB (○), TSB-Fe (■) and TSB+Fe (●).



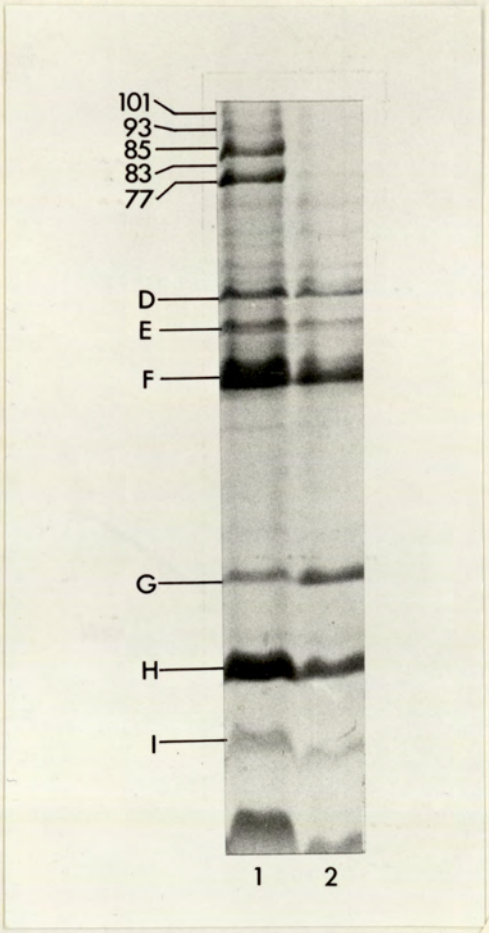
4.2. Analysis of outer membrane protein (OMP) profiles of *P. aeruginosa* cultivated under iron-sufficient and iron-depleted conditions.

The effect of iron-depletion on the OMP profile of *P. aeruginosa* was investigated by SDS-PAGE. Fig. 4.2, i and ii, refers to 2 isolates of *P. aeruginosa* (PAEW and PAGB), and the resulting profiles conformed to the basic pattern described by Mizuno and Kageyama (1978). The nomenclature of the proteins used by these researchers was adopted. In addition, 2 major (77 and 85K) and up to 3 minor (83, 93 and 101K) proteins were evident in the OMs of isolates grown in TSB-Fe (lane 1) which were barely detectable in the OMs of cells cultivated in untreated TSB (lane 2). The only other notable difference between the 2 profiles in both isolates was the apparent reduced expression of protein G in cells cultivated in the iron-depleted media (lane 1) when compared to iron-sufficient cells (lane 2). There was no variation in the OMPs of cells grown in untreated TSB, or Chelex-treated TSB supplemented with 0.02mM $FeSO_4$ (TSB+Fe) (Fig. 4.2, iii, lanes 1 and 2 respectively) indicating that the differences observed in Fig. 4.2 were due specifically to the level of iron in the growth media. In addition, the OMP profile of *P. aeruginosa* PAEW following growth in a chemically defined media lacking iron (CDM-Fe; Fig.4.2, iv) was essentially the same as that seen with TSB-Fe.

The induction of a group of high molecular weight proteins was also observed in the OMs of several other Gram-negative bacteria (*Klebsiella pneumoniae, Proteus mirabilis* and *Escherichia coli*) grown in TSB-Fe (Fig.4.3, lanes 1, 3 and 5). Again, these proteins were largely repressed when the cells were grown under iron-sufficient conditions (lanes 2, 4 and 6). There were no other observable changes in OMPs of these bacteria grown in either media.

The International Antigenic Typing Scheme (IATS) defines 17 O-antigen serotypes of *P. aeruginosa* (Bergan, 1975). Some of these 'O' groups may be

4.2, i.



4.2, ii.

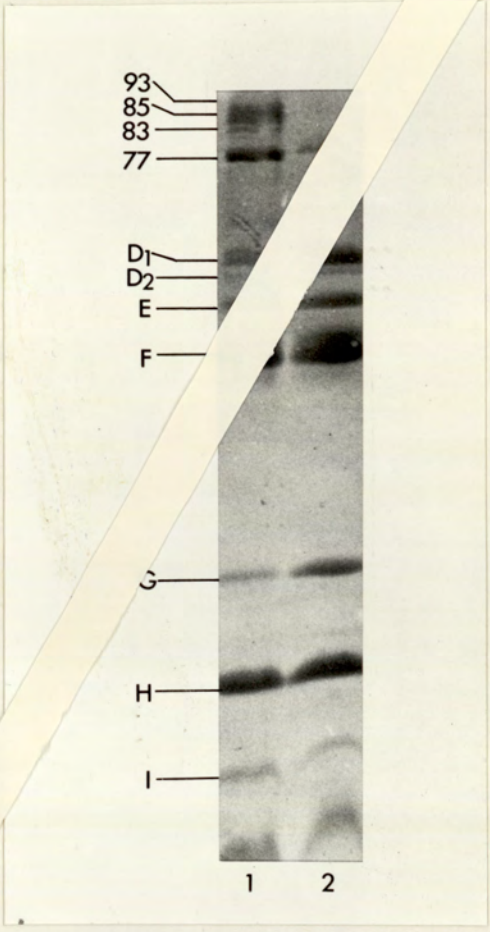
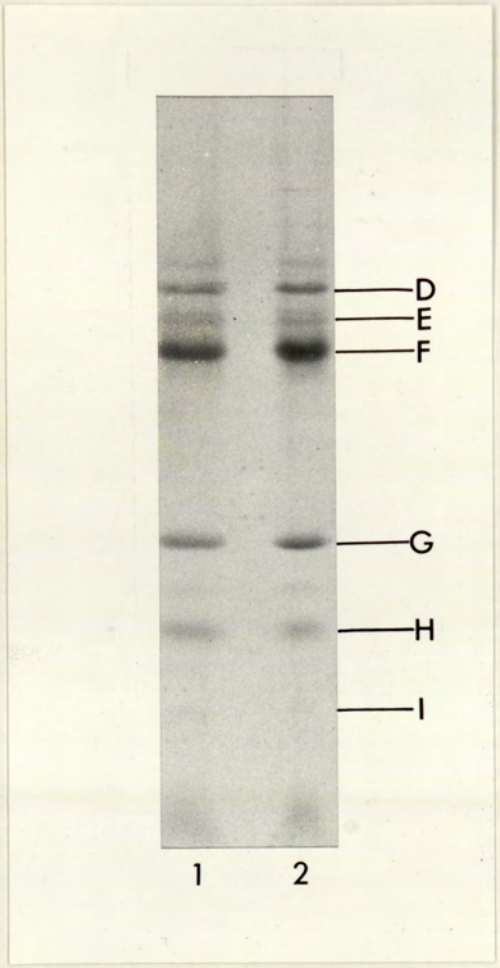


Fig. 4.2, i and ii. Ou[illegible] membrane protein (OMP) profiles of 2 clinical isolates of *P. aerugir*[illegible] cultivated in TSB-Fe (lane 1) and TSB (lane 2). OMPs separated by S[illegible]PAGE (14% acrylamide gel), stained with Coomassie blue and labelled a[illegible]rding to the nomenclature of Mizuno and Kageyama (1978). Numbers ref[illegible] to molecular weight in kilodaltons.

4.2, iii.



4.2, iv.

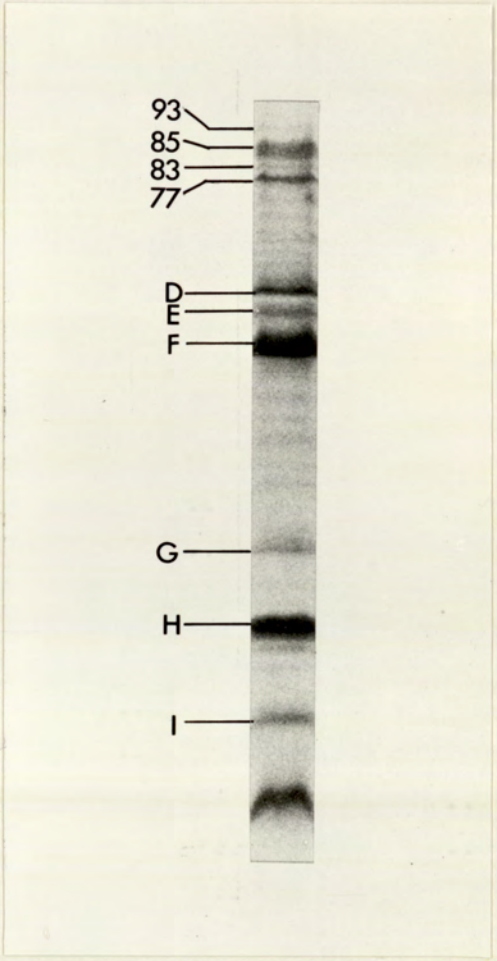


Fig. 4.2, iii. OMP profiles of *P. aeruginosa* cultivated in TSB (lane 1) and chelex-treated TSB supplemented with 0.02mM $FeSO_4$ (TSB+Fe) (lane 2).

Fig. 4.2, iv. OMP profile of *P. aeruginosa* cultivated in CDM-Fe. Molecular weights in kilodaltons.

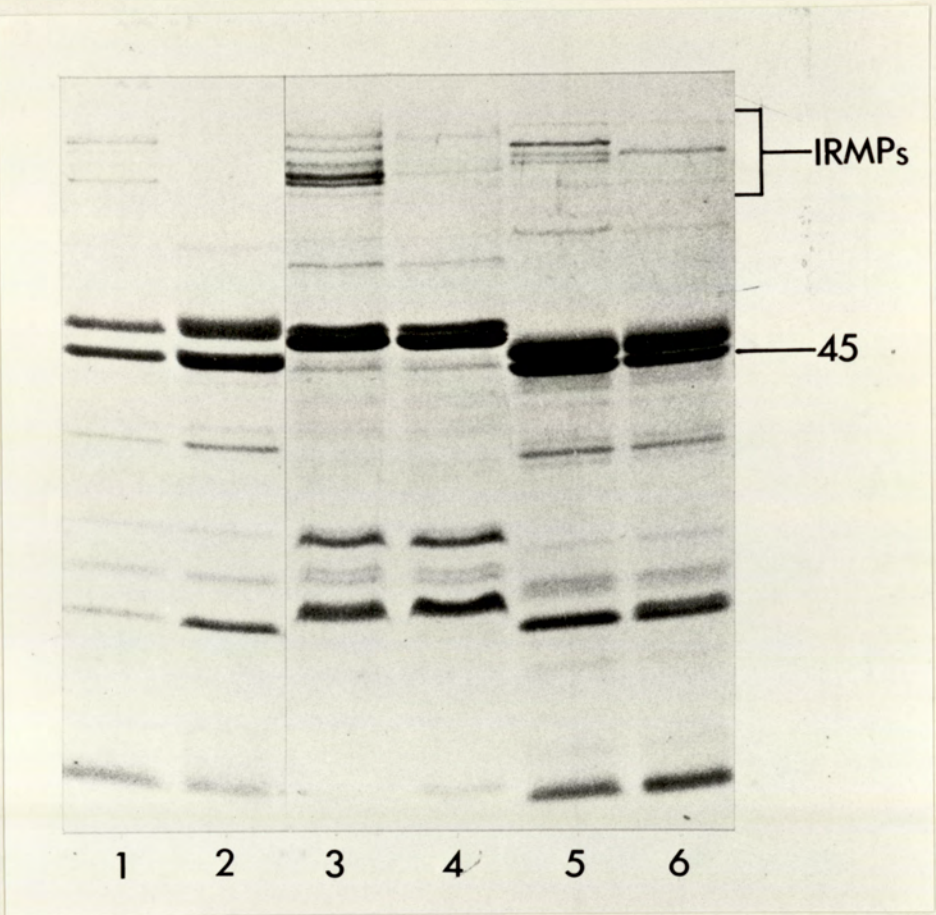


Fig. 4.3. OMP profiles of *K. pneumoniae* (lanes 1 and 2), *P. mirabilis* (lanes 3 and 4) and *E. coli* (lanes 5 and 6) cultivated in TSB (lanes 2, 4 and 6) and TSB-Fe (lanes 1, 3 and 5). Molecular weights in kilodaltons.

subdivided to give a larger number of serotypes. To determine if the high molecular weight iron-regulated membrane proteins (IRMPs) were expressed by members of all 17 serotypes, a representative of each was cultivated under iron-depleted conditions, and the OMPs separated by SDS-PAGE (Fig. 4.4). IRMPs in the region 77 to 101K were evident in all strains, although the degree of expression of the individual proteins varied. Minor differences were also noted for the other major OMPs, particularly with respect to protein G which was present in varying amounts, and in some cases was barely detectable (lanes 7 and 12). However, IRMPs and other OMPs on the whole displayed a high degree of conservation between serotypes.

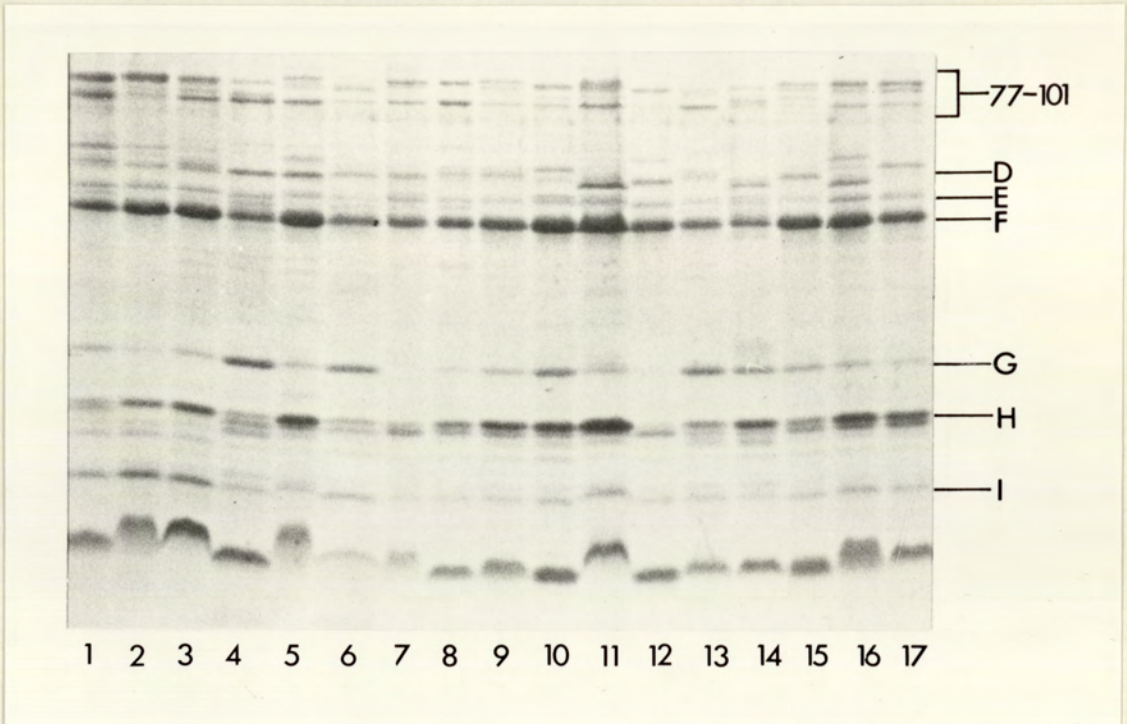


Fig. 4.4. OMP profiles of a representative strain of each of the 17 IATS serotypes of *P. aeruginosa* (lanes 1 to 17 respectively) cultivated in CDM-Fe. Molecular weights in kilodaltons.

4.3. Analysis of LPS extracted from *P. aeruginosa* cultivated under iron-sufficient and iron-depleted conditions.

4.3.1. LPS yields following extraction.

LPS was extracted from *P. aeruginosa* (PAGB, serotype 0:6) using the method described in section 3.2.4. Stationary phase cells cultivated in TSB-Fe and in TSB were used for the extraction and yields of 2.5% and 2.2% respectively were obtained. The figures represent the dry weight of LPS expressed as a percentage of the dry weight of whole cells. Each value was the mean of 3 determinations and the absence of nucleic acids and protein in the purified product was confirmed by lack of absorption at 260nm and 280nm. Hence, there was no significant difference between the yields of LPS from cells grown under iron-sufficient or iron-depleted conditions.

4.3.2. 2-keto 3-deoxy-D-manno-2-octulosonic acid (KDO) content of LPS.

KDO content was determined by the method of Osborn (1963) with slight modifications (section 3.4.2). Values of 42µg and 41.5µg per mg LPS extracted from cells cultivated under iron-depleted and iron-sufficient conditions respectively, were obtained (mean of 3 determinations per sample).

4.3.3. Characterization of LPS by SDS-PAGE and silver stain.

Extracted LPS was analyzed by SDS-PAGE using 14% acrylamide gels and silver-stained according to the procedure of Tsai and Frasch (1982). The results are shown in Fig. 4.5. The LPS was seen to have separated into a series of sharp bands with enhancement of specific regions, indicating a considerable degree of heterogeneity in the size of individual LPS molecules. The heavily stained band at the leading edge of the gel is thought to be rough LPS (lipid A and core) containing no 'O'-antigen, whilst the remaining bands represent LPS molecules possessing increasing numbers

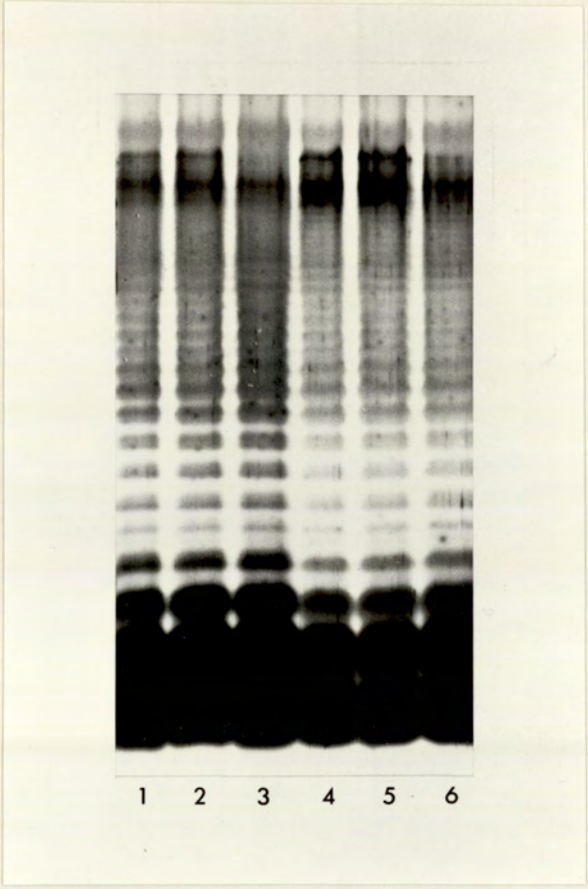


Fig. 4.5. LPS extracted from stationary phase *P. aeruginosa* serotype 0:6 separated by SDS-PAGE and silver-stained by the method of Tsai and Frasch (1982). Lanes 1, 2 and 3 represent loadings of 10, 15 and 20μg LPS extracted from cells cultivated in TSB-Fe and lanes 4, 5 and 6, identical loadings of LPS from cells cultivated in TSB.

of repeating units in their 'O'-side chains, so decreasing their migration rate (Goldman and Leive, 1980; Palva and Mäkëla, 1980). Heterogeneity in the molecular size of LPS from many Gram-negative bacteria has been well-documented (Goldman and Leive, 1980; Palva and Mäkëla, 1980; Tsai and Frasch, 1982; Hitchcock and Brown, 1983; Peppler, 1984). The profile obtained for *P. aeruginosa* O:6 was similar to that reported by MacIntyre *et al* (1986b) for the same serotype. They used 2 methods to extract the LPS, the classic Westphal procedure (Westphal and Jann, 1965) which may select for smooth type LPS, and the method described by Darveau and Hancock (1983) which was shown to extract both smooth and rough forms equally. The LPS purified in this study appeared to contain the full range of LPS species, including a high proportion of rough LPS as well as molecules with extended 'O' side chains. Increasing the concentration of SDS in the separating gel to 0.5% w/v did not alter the profiles obtained (data not shown) indicating that the high molecular weight material observed did not represent aggregates of incompletely dissociated lower molecular weight LPS species, as suggested for some enterobacterial strains (Peterson and McGroarty, 1985). There seemed to be no significant difference between the profiles obtained for LPS extracted from cells grown under iron-sufficient (lanes 1, 2 and 3) or iron-depleted conditions (lanes 4, 5 and 6).

4.3.4. Analysis of cellular and LPS fatty acid composition.

The results of the fatty acid analysis of whole cells and extracted LPS of *P. aeruginosa* are shown in Table 4.1. The fatty acids were identified by retention time comparisons to standards. Integration readings were calculated for each peak and expressed as a percentage of the integration reading obtained for all the peaks in the sample.

Stationary phase whole cells grown under iron-sufficient and iron-depleted conditions were analyzed following acid and alkaline hydrolysis.

Table 4.1. Fatty acid analysis of *P. aeruginosa* whole cells and extracted LPS from cells cultivated in TSB and TSB-Fe.

	WHOLE CELLS				LPS	
	ALKALINE HYDROLYSIS		ACID HYDROLYSIS		ACID HYDROLYSIS	
Fatty Acid	TSB	TSB-Fe	TSB	TSB-Fe	TSB	TSB-Fe
11:0	0.4	0.7	0.6	2.1	0.6	0.7
3OH 10:0	2.5	2.8	3.6	5.9	12.0	9.8
12:0	3.6	6.9	5.2	4.7	26.8	23.9
13:0	0.4	0.2	0.6	0.6	-	-
2OH 12:0	2.5	2.2	5.2	4.4	19.8	21.2
3OH 12:0	0.4	0.7	6.7	5.2	31.8	33.3
14:0	-	-	-	-	0.4	0.5
16:1	7.7	6.4	3.8	2.4	-	-
16:0	35.8	35.7	40.3	44.2	1.5	2.8
$17:0_{cyc}$	1.7	1.7	-	-	-	-
18:1	38.6	35.8	22.4	18.5	-	-
18:0	-	-	2.0	3.9	-	-
$19:0_{cyc}$	3.9	4.1	-	-	-	-
Unknown	-	-	-	-	5.7	5.6

Numbers to left of colon refer to the number of carbon atoms; numbers to right refer to the number of double bonds; cyc refers to a cyclopropane acid; OH refers to a hydroxyl group.
Values represent the percentage of the total fatty acids in the sample

The fatty acid composition of these cells was closely similar to that found by Ikemoto *et al* (1978), Moss (1978) and Lambert and Moss (1983). The predominant fatty acids included 12:0, 16:0, 16:1 and 18:1 with a smaller proportion of 3OH 10:0, 2OH 12:0 and 3OH 12:0. Following alkaline hydrolysis, 2 cyclopropane acids (17:0cyc and 19:0cyc) were also evident. These fatty acids are characteristic of this organism and two other fluorescent pigmented species, *P. putida* and *P. fluorescens*, and their presence enables these bacteria to be distinguished from other *Pseudomonas* species (Moss, 1978). The cyclopropane acids were not found in cells following acid hydolysis, a finding also reported by Lambert and Moss (1983). This probably reflects degradation of these fatty acids by the rigorous hydrolysis procedure used. Compared to alkaline hydrolysis, an 8 to 10 fold increase in the relative percentage of 3OH 12:0 was observed following acid hydrolysis. This hydroxy fatty acid is amide-linked to the glucosamine backbone of lipid A in LPS whereas most other fatty acids are ester-linked (Wilkinson, 1983). Consequently, acid conditions are required for its full liberation (Lambert and Moss, 1983). There appeared to be a reduction in the percentage of 16:1 and 18:1 following acid hydrolysis, also reported by Lambert and Moss (1983), but the relevance of this is unknown. Minor and probably insignificant differences between the relative proportions of certain fatty acids (11:0, 18:0) in cells grown under iron-sufficient or iron-depleted conditions were observed.

Extracted LPS was analyzed using acid hydrolysis to achieve complete liberation of the fatty acids amide-linked to glucosamine. The main constituents were found to be 12:0, 2OH 12:0, 3OH 12:0, 3OH 10:0 and a small proportion of 16:0. There were no unsaturated fatty acids present indicating that the LPS was not contaminated with phospholipids (Darveau and Hancock, 1983). Similar constituents and relative percentages have been described by other researchers (Drewry *et al*, 1973; Wilkinson and Galbraith, 1975;

Kropinski *et al*, 1985). An additional peak which eluted between 12:0 and 2OH 12:0 and accounted for about 5% of the total fatty acids, was observed. The retention time of this peak did not correlate with any of the standard fatty acids, hence it could not be identified. It may represent an artefact, as a result of the degradative effects of acid hydrolysis on other components (Lambert and Moss, 1983). Again, there were no major differences between the fatty acid composition of LPS extracted from cells grown under iron-sufficient or iron-depleted conditions.

4.4. Discussion.

Studies have shown that nutrient depletion has a marked effect on envelope structure and related properties of bacteria (Costerton *et al*, 1979; Brown and Williams, 1985 a and b). Iron is known to be essential for bacterial proliferation and it is now clearly recognized that restriction of freely available iron is an important non-specific host defence mechanism against infection (Bullen, 1981; Griffiths, 1983). Furthermore, there is considerable evidence that bacteria in human infections grow under iron-restricted conditions (Brown *et al*, 1984; Lam *et al*, 1984; Shand *et al*, 1985). Bacteria used in laboratory research have traditionally been cultivated under iron-plentiful conditions in complex or simple salts media. Growth in media where levels of freely available iron are low, may more closely resemble the situation *in vivo*. Methods involved in the removal or restriction of trace metals from culture media are diverse (Waring and Werkman, 1942; Donald *et al*, 1952). They include the use of spent media, recrystallization, precipitation or absorption, all of which have associated problems. Alternatively, synthetic chelating agents may be added, but such chemicals may damage or alter other bacterial properties (Klebba *et al*, 1982). Chart *et al* (1986) reported that in *E. coli* the nature of the iron chelator added to the medium affected the expression of IRMPs. Both quantitative and qualitative differences in the IRMPs were noted as well as changes in the growth kinetics of the organism, highlighting the problems of this method of iron-restriction. Such compounds may chelate metal ions other than iron which perhaps accounts for some of the differences, or the compounds themselves may affect other cellular functions. A synthetic iron chelator reported to resemble biological chelators, desferroxamine, has been used (van Asbeck *et al*, 1983). Iron chelators of biological origin restrict iron availability (Griffiths, 1983) with the benefit of more closely

reflecting conditions *in vivo*, but they are expensive and require extensive dialysis prior to use.

However, treatment of TSB with the ion-exchange resin Chelex-100 followed by replacement of other metal cations was shown to remove sufficient iron from the media to impose conditions of iron-deprivation on a range of Gram-negative bacteria such that bacterial yields were reduced and a number of high molecular weight proteins were induced in the OM. In *P. aeruginosa*, up to 5 additional proteins were observed following one-dimensional SDS-PAGE. IRMPs were also shown to be expressed by representatives of all 17 IATS serotypes when grown under similar iron-depleted conditions. Two-dimensional SDS-PAGE, which separates proteins according to their isoelectric point in the first dimension and by molecular weight in the second dimension (O'Farrell, 1975), was found further to resolve the IRMPs into a cluster of 8 or more proteins (data not shown). In response to iron deprivation *P. aeruginosa* is known to produce several endogenous iron scavenging compounds or siderophores (Cox and Graham, 1976; Cox and Adams, 1985) which can acquire iron from host chelators such as transferrin. It is assumed that at least one of the high molecular weight IRMPs acts as a receptor for these iron-siderophore complexes so enabling uptake of iron into the cell. Similar receptors have been identified in other bacteria. For example, in *E. coli* an 81K (fep A) protein was shown to function as the receptor for ferric enterobactin (Icihara and Mizushima, 1978) and Magazin *et al* (1986) genetically demonstrated that an 85K protein was the receptor for ferric pseudobactin in a fluorescent *Pseudomonas* strain B10. More recently, Cody and Gross (1987) reported that ferric pyoverdin required a 74K protein for uptake into cells of *Pseudomonas syringae* pv. *syringae*.

Another effect of iron-deprivation in *P. aeruginosa* appeared to be the reduced expression of protein G in the OM. Nicas and Hancock (1980)

have reported that Mg-limited and NH_4^+-limited cells of *P. aeruginosa* also showed reduced expression of this protein although its function has not been defined. Further work is required to determine the nature and control of expression of protein G.

Several papers have reported the induction of an additional 14K protein in *P. aeruginosa* when cultivated under conditions of iron-depletion, which the researchers claim specifically binds [Fe^{59}]-pyochelin (Sokol and Woods, 1983, 1984 and 1986). A similar protein has not been observed in this and other studies from our laboratory (Anwar *et al*, 1984; Shand , 1985) which have included investigation of a large number of wild type and laboratory strains grown under iron-depletion. It is possible that differences in the methodology employed for OM preparation between the 2 groups may contribute to the discrepancy (Chopra and Shales, 1980). However, several experimental points in the protocol of Sokol and Woods raise certain doubts about their conclusions. A valid comparison between iron-plentiful and iron-deprived cells may not have been made by these researchers (Sokol and Woods, 1983). SDS-PAGE gels comparing iron-plentiful and iron-limited preparations were not shown but laser densitometer scans indicated high molecular weight proteins (≃75-100K) present in both preparations. Furthermore, OMs were incubated with [^{59}Fe]-pyochelin and denatured with 2% SDS before SDS-PAGE analysis and subsequent autoradiography to establish where the [^{59}Fe]-pyochelin had bound. It is unlikely that the binding of ^{59}Fe to pyochelin or of [^{59}Fe]-pyochelin to receptor protein would withstand SDS-denaturation unless the links between molecules were covalent. There is no evidence to suggest that this is so. No SDS-PAGE gels of [^{59}Fe]-pyochelin running alone were shown. Discrepancies were also noted in the [^{59}Fe]-pyochelin binding values stated in 2 of the studies (Sokol and Woods, 1983 and 1984). The entity running at 14K was not fully characterized in any of the published papers (Sokol and Woods,

1983, 1984 and 1986; Sokal, 1984) and there is no direct evidence that the protein plays a part in transport of [^{59}Fe]-pyochelin in intact cells of *P. aeruginosa*. The protein itself was subsequently purified from whole cells (Sokol and Woods, 1984) not OM fractions as previously used in the binding studies (Sokol and Woods, 1983). A 14K protein has been reported in periplasmic fractions of iron-deprived *P. aeruginosa* (Cox, 1985) and it is possible that the antigen described by Sokol and Woods corresponds to this periplasmic protein. Finally, the 14K band may represent a fragment of a larger protein which binds [^{59}Fe]-pyochelin. Indeed, Sokol (1987), on performing monoclonal antibody affinity chromatography studies with antibodies to the 14K species found a high molecular weight form of the ferripyochelin binding protein at 116K, which may support this theory. Whether such a protein corresponds to any of the IRMPs described in this study remains to be investigated.

The extracted LPS was analyzed by SDS-PAGE and silver-staining and was seen to be resolved into a multibanded ladder pattern, characterized by regions of greater intensity. It is thought that different molecular species of LPS are dissociated into monomolecular units through binding of SDS to the lipid A (Olins and Warner, 1967). The silver stain is very sensitive (Tsai and Frasch, 1982), detecting nanogram quantities of LPS. It has been suggested that the staining is dependent upon the presence of periodate-sensitive cis-glycols in the core and side-chain polysaccharide of the LPS molecules (Tsai and Frasch, 1982). However, Kropinski *et al* (1986) subsequently demonstrated that polysaccharide fractions released from LPS by acid hydrolysis were not stained by silver, whilst the lipid A fraction and whole LPS did react with the stain. Hence, the exact basis of the silver stain is complex and remains unclear.

The LPS extracted from cells cultivated under iron-depleted or iron-sufficient conditions demonstrated similar profiles on analysis by SDS-

PAGE. KDO determinations also revealed no significant differences. Finally, despite knowledge that other environmental parameters such as media components, pH and growth temperature may affect the fatty acid composition of bacteria (Lechevalier, 1977; Kropinski *et al*, 1987) both cellular and LPS fatty acids were essentially the same in iron-depleted and iron-sufficient cells.

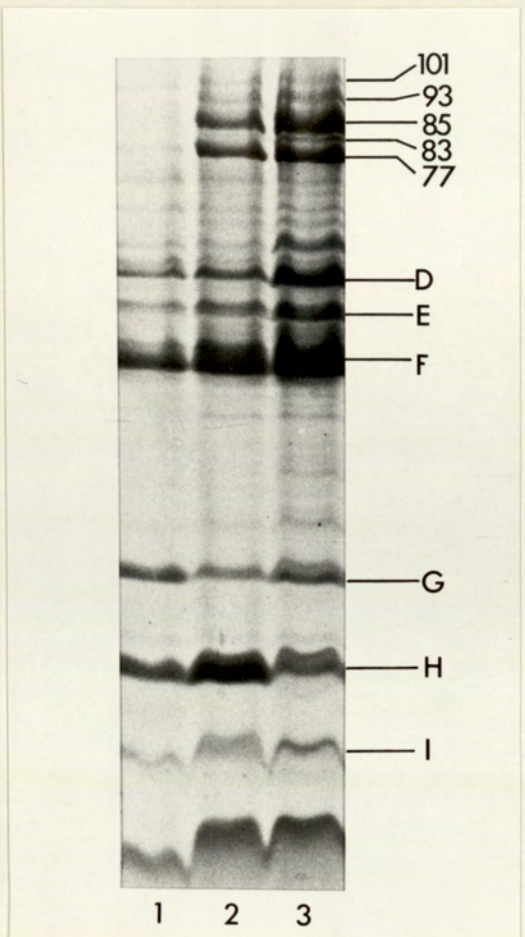
5. Serum and local antibody response to OM antigens of *P. aeruginosa* isolated directly from human burn wounds.

5.1. OM antigens expressed *in vivo* by *P. aeruginosa* in burn wounds.

P. aeruginosa was isolated directly, without subculture, from the infected wound tissue of 2 patients with severe burn injury. The first patient (female, age 76) was previously fit and healthy but suffered a full thickness burn to the left elbow. The wound became infected 14 days post burn, and 6 days later wound excision was performed and infected tissue obtained. Microbiological analysis of the tissue revealed *P. aeruginosa* serotype 0:4 (PAEW) as the sole infecting organism. The second patient (female, age 85) was admitted with 11% burns to the back of the scalp and shoulders with 4% full thickness burns to the shoulder. 10 days postburn the wound showed signs of infection (green, foul-smelling dressings) and 8 days later the wound was excised, and infected tissue obtained. Microbiological analysis indicated *P. aeruginosa* serotype 0:6 (PAGB) as the predominant infecting organism (80%). Silver-sulphadiazine dressings were initially applied, then changed to chlorhexidine dressings and after 5 weeks the infection had resolved. The isolates from the two patients were cultivated *in vitro* in TSB under iron-sufficient and iron-depleted conditions.

Figs. 5.1, i and 5.1, ii, refer to patients 1 and 2 respectively. They show the outer membrane protein (OMP) antigens of *P. aeruginosa* PAEW (i) and *P. aeruginosa* PAGB (ii) obtained directly from the wound tissue (lane 3) and the same isolates grown in TSB-Fe (lane 2) and TSB (lane 1), separated by SDS-PAGE and stained with Coomassie brilliant blue. The resulting profiles again conformed to the basic pattern described by Mizuno and Kageyama (1978). Proteins D, E, F, G, H and I were all expressed

5.1, i.



5.1, ii.

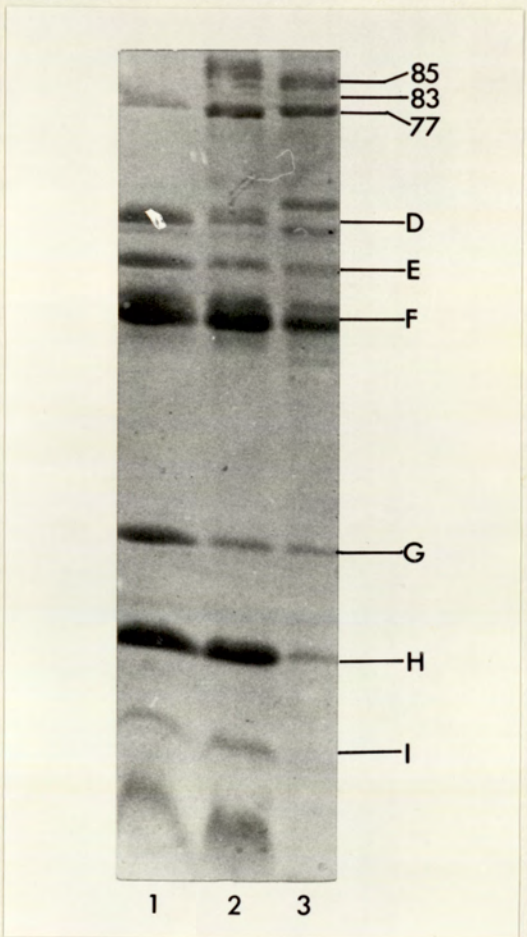


Fig. 5.1, i and ii. OMP profiles of *P. aeruginosa* PAEW (i) and P. aeruginosa PAGB (ii) recovered directly from infected burn wound tissue (lane 3) and the same isolates cultivated in TSB-Fe (lane 2) and TSB (lane 1). Molecular weights in kilodaltons.

in the OM of *in vivo* and *in vitro* grown cells (I was less apparent in the *in vivo* OMs of *P. aeruginosa* PAGB; 5.1, ii, lane 3). A further protein of approximate molecular weight 55K, was also present, more noticeably in the *in vivo* OMs of *P. aeruginosa* PAEW (5.1, i, lane 3). Additionally, several high molecular weight iron-regulated membrane proteins (IRMPs, 77-101K) were induced in the OMs of the isolates cultivated in TSB-Fe (lane 2) which were barely detectable in the OMs of cells cultivated in TSB (lane 1). The strong expression of these proteins was also evident in the OMs of cells isolated directly from the infected wound tissue (lane 3). The presence of such proteins provides direct evidence that *P. aeruginosa* grew under iron-restricted conditions in the burn wounds of these two patients. Additional minor differences in the protein bands of *in vivo* and *in vitro* grown cells were observed, more particularly for patient 2 (Fig. 5.1, ii). This may suggest that other environmental factors for example pH, osmolarity or growth on an epithelial surface may affect the expression of OMPs *in vivo*.

Proteinase-K digestion of the above OM preparations to destroy proteinaceous material (Hitchcock and Brown, 1983), followed by SDS-PAGE and silver-staining (Tsai and Frasch, 1982) enabled the lipopolysaccharide (LPS) profiles of these bacteria to be studied (Fig. 5.2, i and ii). For patient 1 (5.2, i) the ladder patterns obtained were similar in all cases, although it would appear that the LPS of *in vivo* bacteria (lanes 3 and 4) included bands of slightly higher molecular weight (marked with arrow) than in the corresponding *in vitro* grown isolates (lanes 1 and 2). These may represent a proportion of LPS molecules with longer 'O'-polysaccharide side chains. For patient 2 (Fig. 5.2, ii) *in vivo* and *in vitro* profiles showed a clearer difference, with variations in the interband spacing as well as higher molecular weight material present in cells direct from the wound. Kuzio and Kropinski (1983) proposed that a shift to a higher molecular

5.2, i.

5.2, ii.

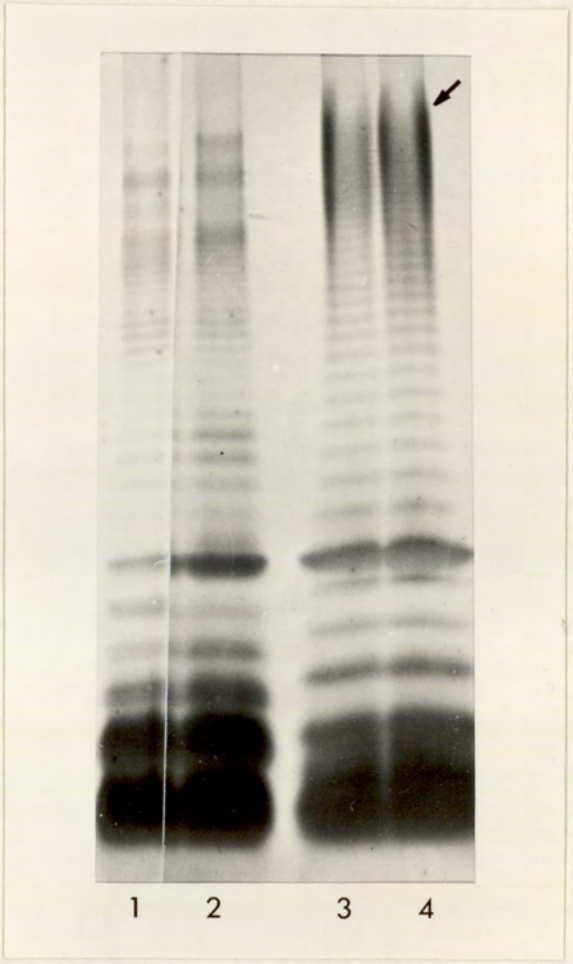


Fig. 5.2, i and ii. Proteinase-K digests of OMs from *P. aeruginosa* PAEW (i) and *P. aeruginosa* PAGB (ii) recovered directly from infected burn wound tissue (lanes 3 and 4) and the same isolates cultivated in TSB-Fe (lane 2) and TSB (lane 1). Resulting LPS profiles were stained with silver. Arrows indicate higher molecular weight LPS bands.

weight such as that observed could be indicative of a real increase in molecular weight of the side chain (since the core regions migrate to the same position), or a decrease in the net negative charge on the LPS molecules. In addition, Pluschke *et al* (1986) noted that altered LPS migration patterns correlated with variations in chemical composition, but further suggested that a combination of size and hydrophobicity may affect the resolution of LPS by SDS-PAGE.

5.2. Humoral immune response to *P. aeruginosa* OM antigens in burn wound infection studied by immunoblotting.

5.2.1. Serum and local antibody response to OMPs of *in vivo* and *in vitro* grown bacteria.

Immunoblotting combines the high resolution of SDS-PAGE with a solid-phase immunoassay such that the antigenic properties of separated bacterial components can be investigated (Towbin and Gordon, 1984). During the transfer components are concentrated on the surface of the nitrocellulose (NC) paper, freed from denaturing SDS in the gel and in part, renatured (Hjerten, 1983). However, full antigenicity of each antigen may not be restored and the transfer may not necessarily be quantitative for the proteins present on the original gel (Gershoni and Palade, 1982), hence a degree of caution should be taken on interpreting the results.

The separated OMPs shown in Fig. 5.1, i of *P. aeruginosa* PAEW from patient 1 were electrophoretically transferred to NC paper and an amido black stain (Fig. 5.3, i) carried out to determine qualitative transfer of the protein bands. Similar blots were then reacted with antibodies present either in the serum or locally in wound tissue fluid from this patient. The recognition of OM antigens by serum IgM antibodies 1 week after onset of wound infection (Fig. 5.3, ii) indicated that IRMPs of P. aeruginosa PAEW either cultivated *in vitro* under iron-depleted conditions or obtained directly from the wound were detected, more strongly in the latter case (lane 3). There was no recognition of such proteins in iron-plentiful cells (lane 1). In addition, a response to proteins D, E and H in all 3 lanes was noted along with the 55K antigen in lane 3. F and G were only faintly detected. Subsequent serum samples gave a similar pattern of response (data not shown).

Serum IgG antibodies (Fig. 5.3, iii) also detected IRMPs as well as proteins D and E, but the response to porin protein F was again barely

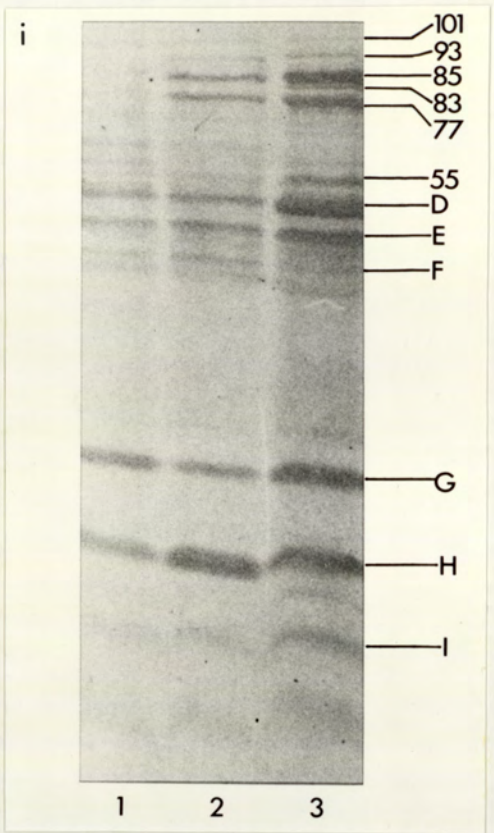


Fig. 5.3, i. OMPs of *P. aeruginosa* PAEW recovered directly from burn wound tissue (lane 3) and the same isolate cultivated in TSB-Fe (lane 2) and TSB (lane 1). OMPs transferred to NC paper and stained with amido black 1% w/v.

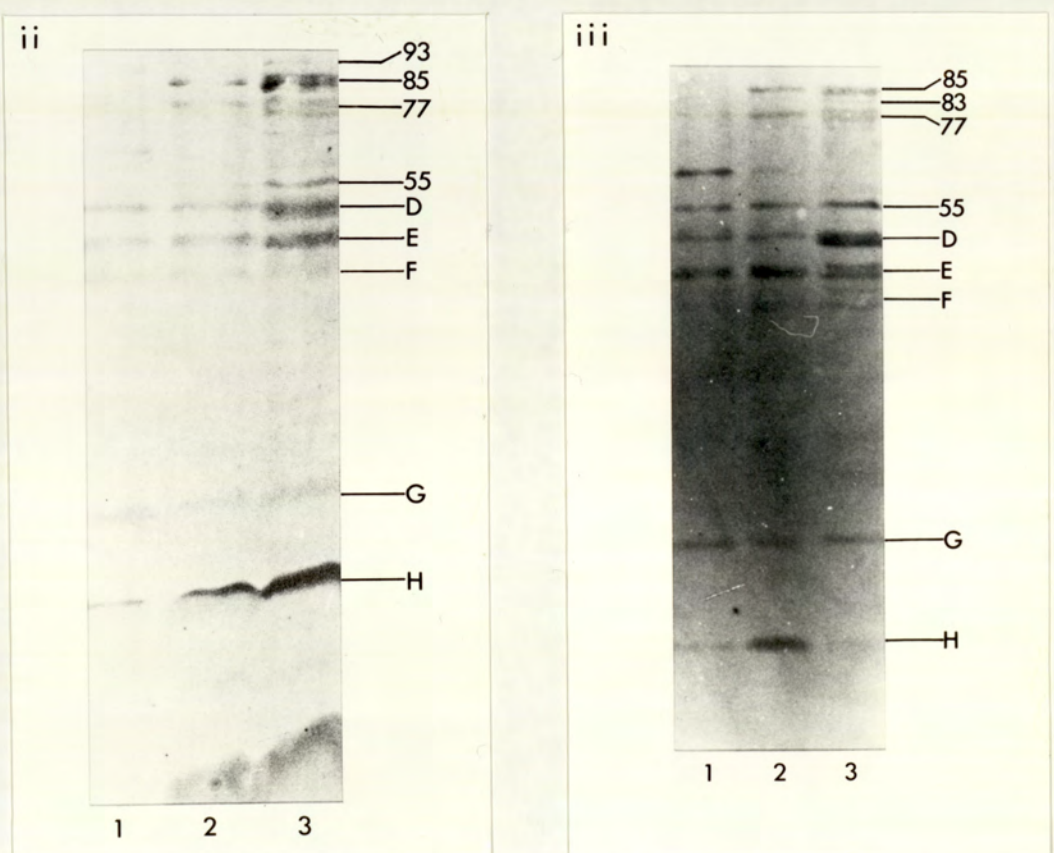


Fig. 5.3, ii and iii. Immunoblot of the OMPs described in Fig. 5.3, i probed with serum from patient 1, followed by peroxidase-labelled anti-human IgM (ii) or IgG (iii). Serum obtained 1 week after onset of infection.

detectable. There was a stronger reaction with the 55K antigen in all 3 lanes. Protein H in the OM of cells grown *in vitro* (lanes 1 and 2) was recognized, particularly in the iron-depleted cells (lane 2), but there appeared to be no corresponding reaction to the protein in the *in vivo* OM preparation (lane 3). No notable variation in the IgG response was observed in following weeks.

IgA antibodies in serum obtained 1, 2 and 4 weeks after onset of infection only faintly recognized the IRMPs initially, but during the course of the infection, the proteins elicited a stronger response (Fig. 5.3, iv, a, b and c). The reaction to proteins D, E and H remained the same. However, as with the IgG response, protein H was only detected in the OMP profile of *in vitro* grown cells (lanes 1 and 2) not that of *in vivo* grown cells (lane 3). This observation is difficult to explain. It may be due to the reduced expression of the protein *in vivo* although reference to the SDS-PAGE profile (Fig. 5.1, i, lane 3) and to the amido black stain (Fig. 5.3, i, lane 3) indicates that the protein is present in detectable amounts in the OMs of cells direct from the wound. It perhaps reflects antigenic variation, certain epitopes which elicited antibody formation *in vivo* being lost following the procedure used to extract bacteria directly from the burn wound. An alternative explanation, in view of the apparent recognition of this band by IgM (Fig. 5.3, ii, lane 3) is that IgM molecules have a higher affinity for epitopes on the protein than immunoglobulins of different classes (IgG and IgA). Consequently, IgM may bind to the exclusion of IgG or IgA.

The molecular weight (55K) of the antigen evident in the Coomassie blue-stained profiles (Fig. 5.1, i) and detected by antibodies of all classes suggested that it may be flagella protein. To investigate this further, flagella were extracted and purified from *P. aeruginosa* PAEW using the method of Montie *et al* (1982a) and shown to migrate as a single band of

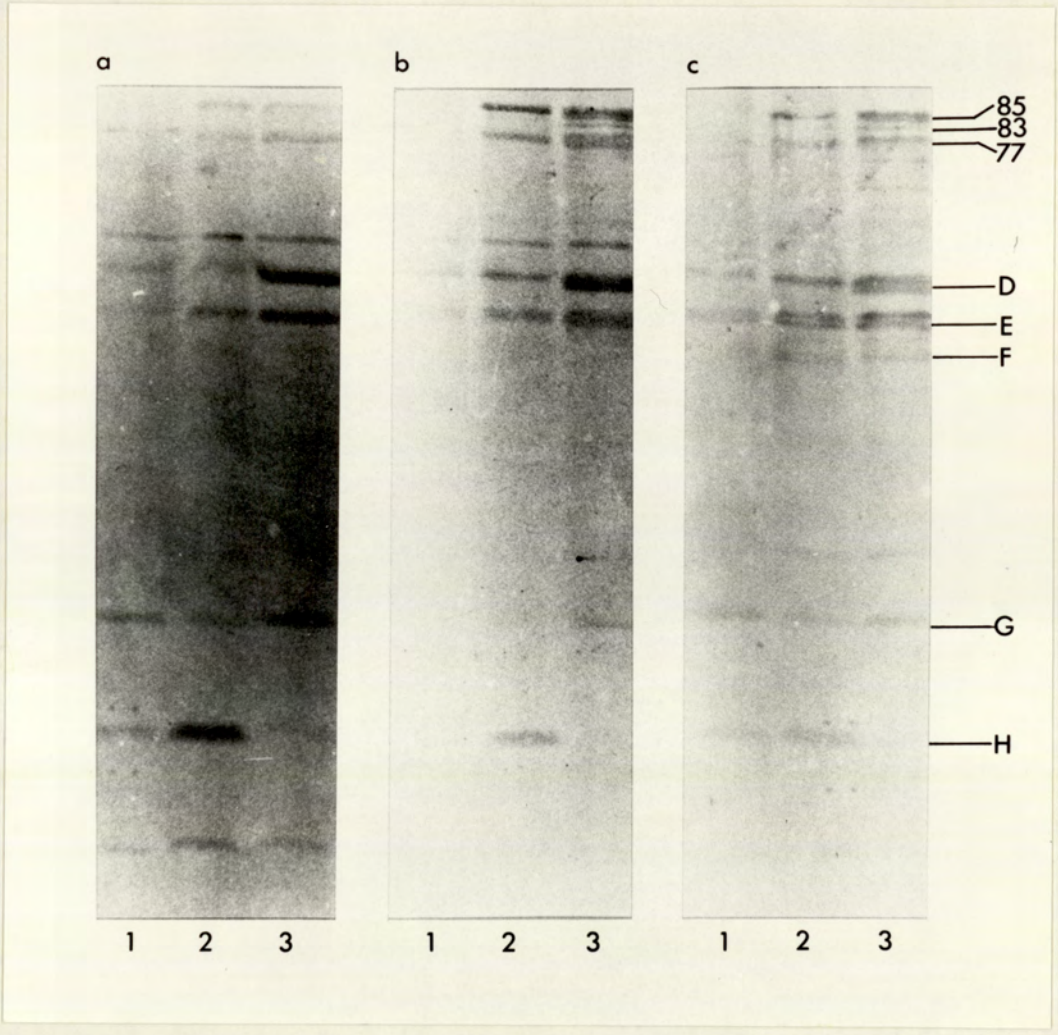


Fig. 5.3, iv. Immunoblots of the OMPs described in Fig. 5.3, i, probed with serum from patient 1, followed by peroxidase-labelled anti-human IgA.

Serum obtained 1, 2 and 4 weeks after onset of infection (a, b and c respectively).

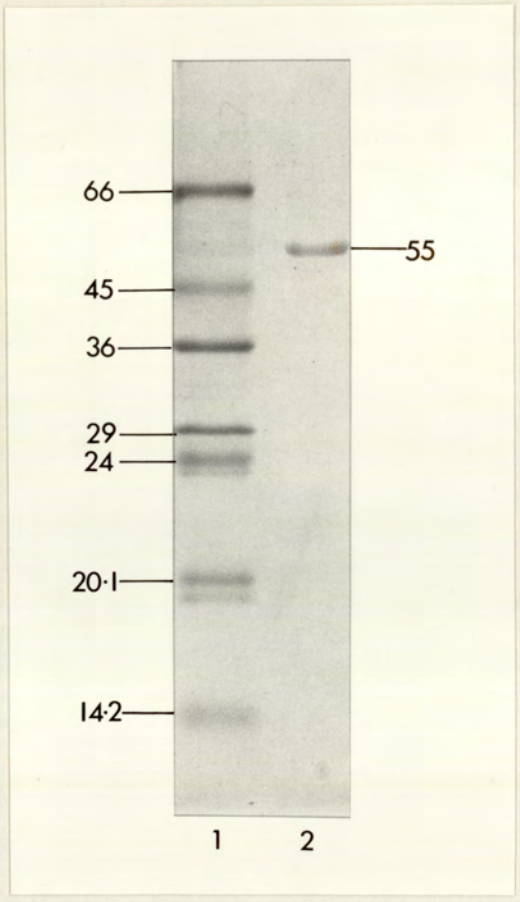
Molecular weights in kilodaltons.

approximate molecular weight 55K when analyzed by SDS-PAGE (Fig. 5.4, i). Fig. 5.4, ii, demonstrates the purified antigen transferred to NC paper and probed with patient serum. There was a marked reaction to the protein indicating that it had elicited a strong antibody response during the course of the infection.

To determine the presence of immunoglobulins locally in the vicinity of the burn wound, the OM antigens shown in Fig. 5.1, i, following transfer to NC paper were probed with tissue fluid retained after isolating the bacteria, and concentrated by lyophilization (section 3.2.5). Figs. 5.5, i, ii and iii, represent the IgM, IgG and IgA response repectively. There was a marked reaction of IgG antibodies with IRMPs, proteins D, E and H (except lane 3). IgM and IgA antibodies were directed predominantly against the IRMPs with only weak recognition of D and E. The latter observation may reflect competition between immunoglobulin subclasses for the binding sites on these proteins, IgG antibodies having a higher affinity for the epitopes than IgM or IgA. The IgM response was weak in comparison to IgG and IgA which may in part be explained by the size of IgM molecules ($\simeq$ 900K). Hence, they are largely confined to the vascular system (Mims, 1982).

Figs. 5.6 to 5.7 refer to the immunoblots obtained with serum and tissue fluid from patient 2. The reaction with IgM was too weak to be photographed which may suggest that this patient had been in contact with *P. aeruginosa* at some earlier stage, hence this infection resulted predominantly in an IgG response. Fig. 5.6, ii, demonstrates OMPs detected by IgG at 1, 2 and 3 weeks after first signs of infection. As with patient 1, IRMPs and proteins D and E were recognized at all stages. There appeared to be a progressive increase in the reaction to protein F up to the third week indicating a later response to this antigen. Protein H was recognized but again only in the OMs of the *in vitro* grown bacteria. The IgA response

5.4, i.



5.4, ii.

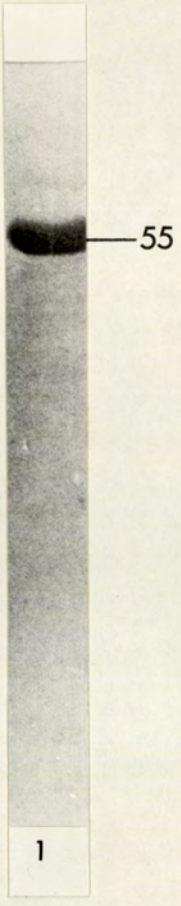


Fig. 5.4, i. SDS-PAGE of purified flagella protein from *P. aeruginosa* PAEW (lane 2) and molecular weight standards (lane 1) as follows: 66K, bovine albumin; 45K, egg albumin; 36K, glyceraldehyde-3 phosphate dehydrogenase; 29K, carbonic anhydrase; 24K, trypsinogen; 20.1K, trypsin inhibitor; 14.2K, α-lactalbumin.

Fig. 5.4, ii. Immunoblot of purified flagella protein electrophoretically transferred to NC paper and probed with serum from patient 1, followed by peroxidase-labelled anti-human IgA.

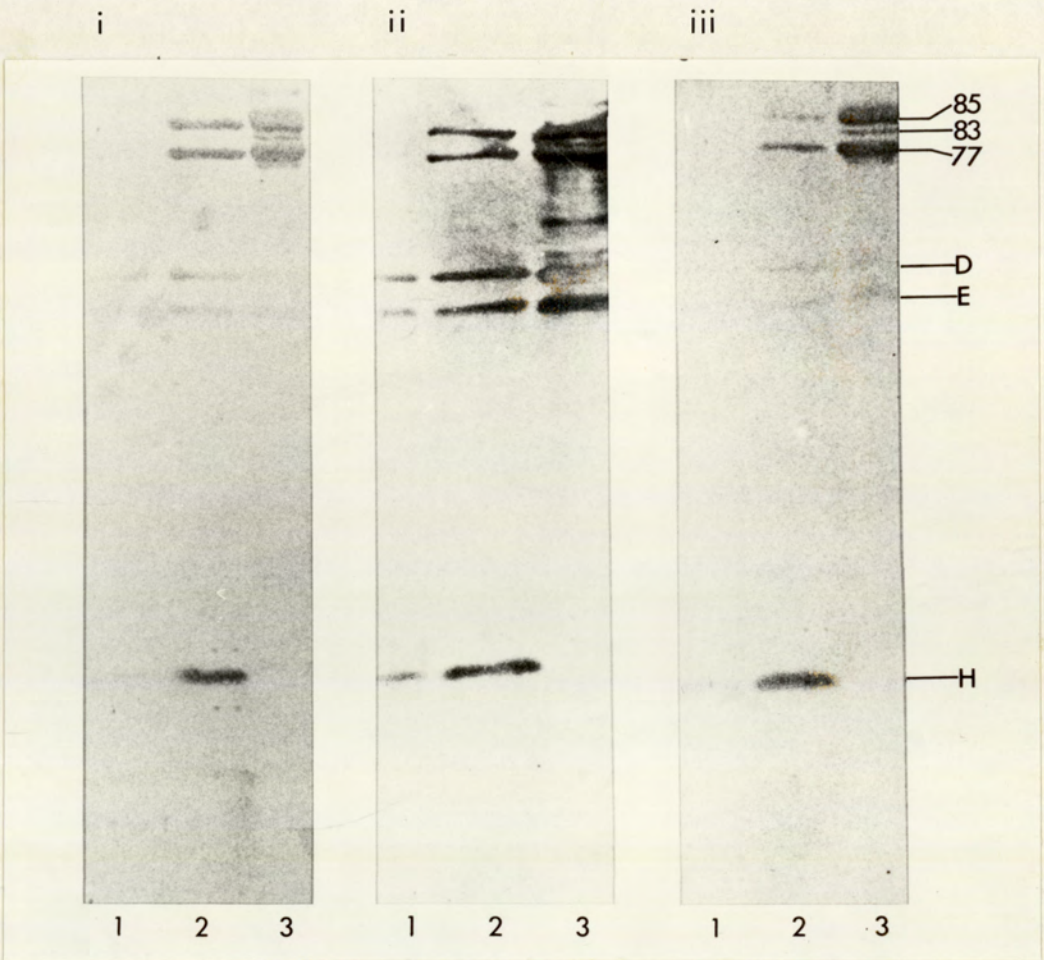


Fig. 5.5, i, ii and iii. Immunoblot of *P. aeruginosa* PAEW OMPs probed with wound tissue fluid from patient 1, followed by peroxidase-labelled anti-human IgM (i), IgG (ii) and IgA (iii). OMPs of cells recovered directly from burn wound tissue (lane 3) or cultivated in TSB-Fe (lane 2) or TSB (lane 1). Molecular weights in kilodaltons.

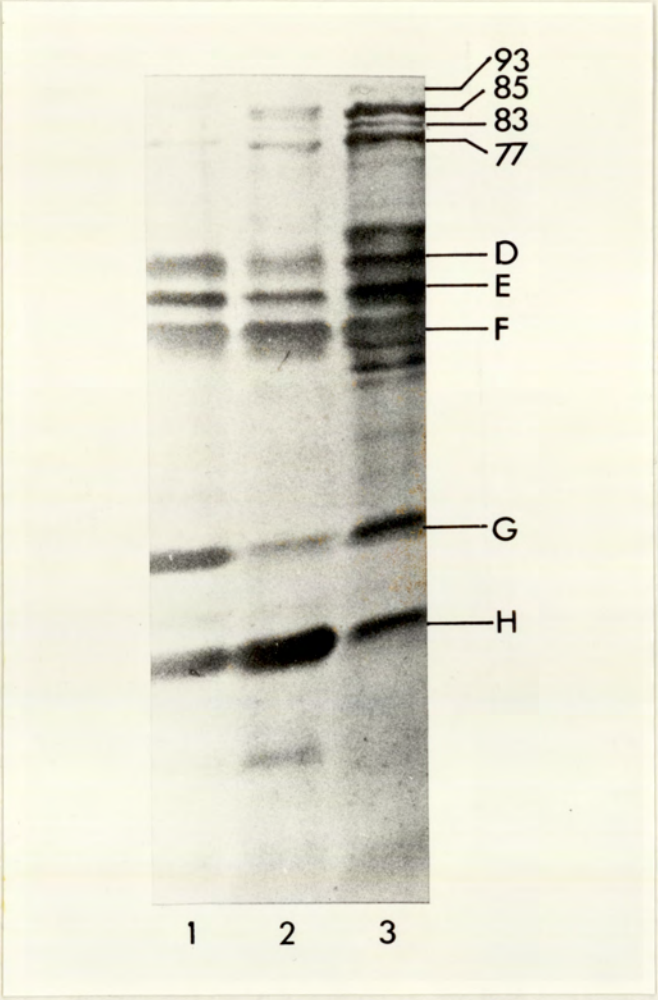


Fig. 5.6, i. OMPs of *P. aeruginosa* PAGB recovered directly from the burn wound tissue of patient 2 (lane 3) or the same isolate cultivated in TSB-Fe (lane 2) or TSB (lane 1). OMPs separated by SDS-PAGE, electrophoretically transferred to NC paper and stained with amido black 1% w/v. Molecular weights in kilodaltons.

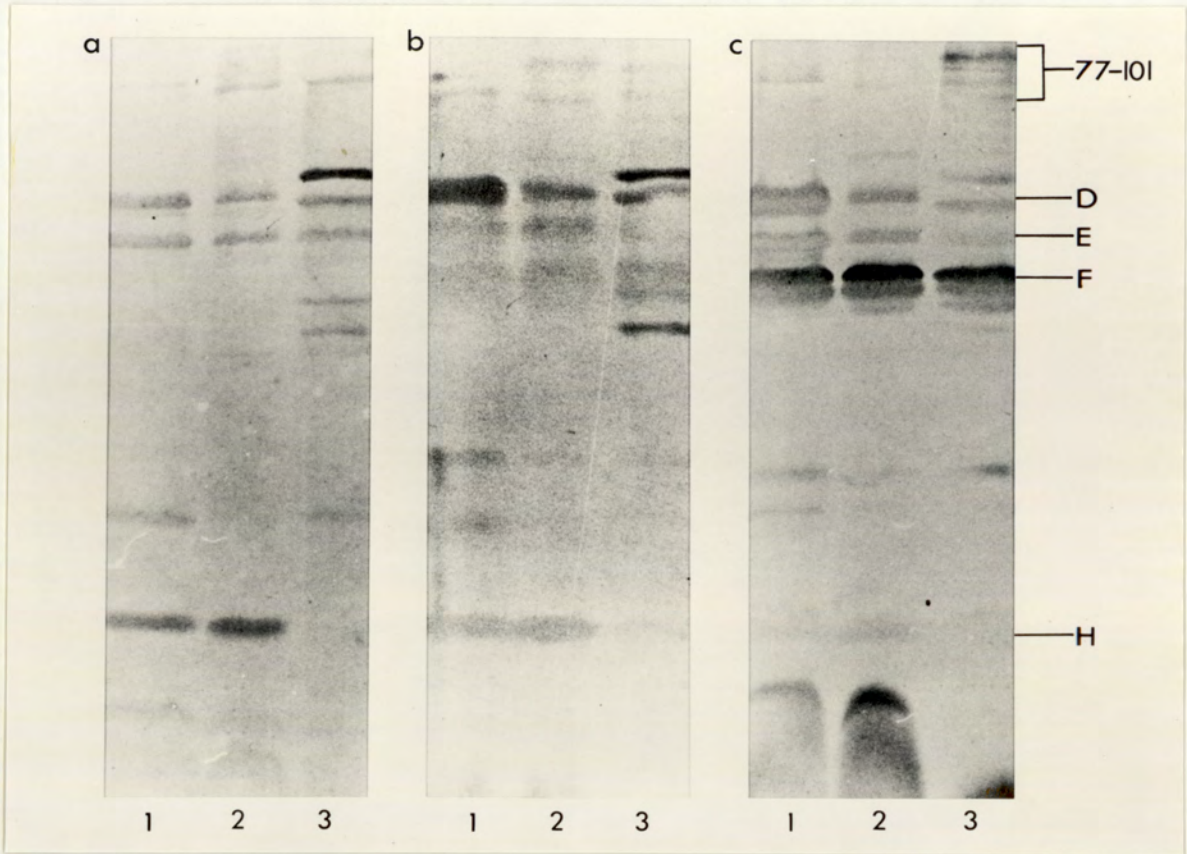


Fig. 5.6, ii. Immunoblot of the OMPs described in Fig. 5.6, i, probed with serum from patient 2 followed by peroxidase-labelled anti-human IgG. Serum obtained 1, 2 and 3 weeks after onset of infection (a, b and c respectively)

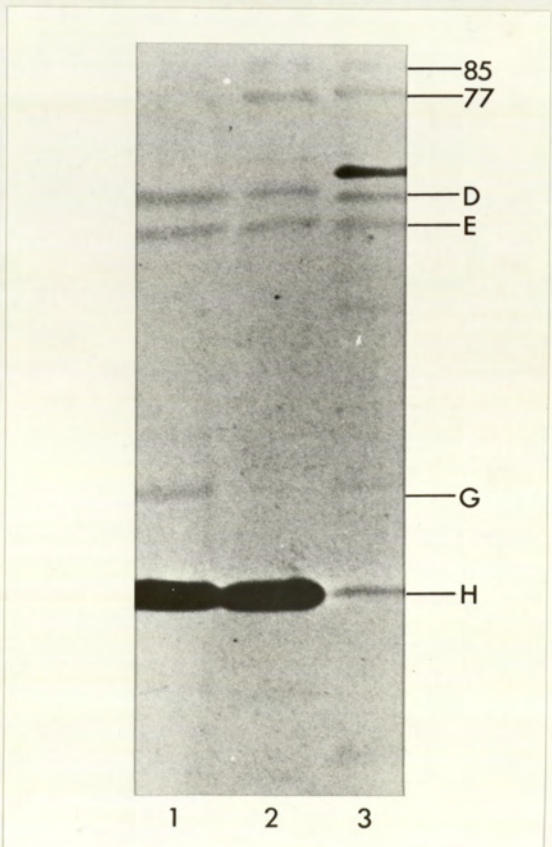


Fig. 5.6, iii. Immunoblot of the OMPs described in Fig. 5.6, 1 probed with serum from patient 2 followed by peroxidase-labelled anti-human IgA. Serum obtained 2 weeks after onset of infection. Molecular weights in kilodaltons.

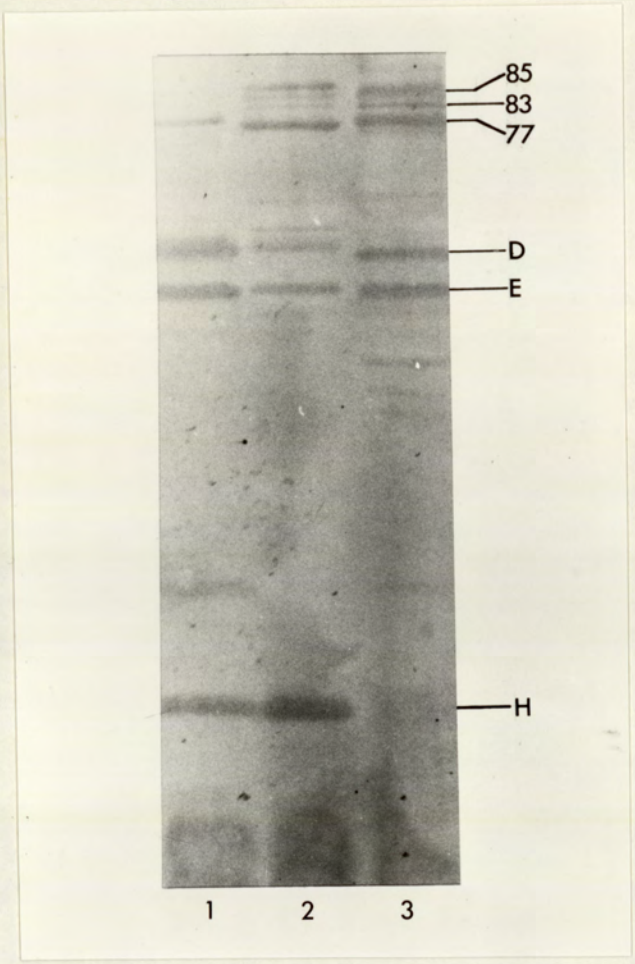


Fig. 5.7. Immunoblot of the OMPs described in Fig. 5.6, i, probed with wound tissue fluid from patient 2, followed by peroxidase-labelled anti-human IgG. Molecular weights in kilodaltons.

(Fig. 5.6, iii) showed a striking reaction to protein H in lanes 1 and 2 with slight recognition of the *in vivo* protein implying a surprisingly high titre of IgA antibodies to the antigen. The reason for this is unknown. There was little response to protein F and this pattern did not change. IgG antibodies in wound tissue fluid detected IRMPs, D, E and H (Fig. 5.7), similar to the reaction seen with patient 1.

Serum was obtained from 2 further patients (3 and 4) with *P. aeruginosa* infection, although it was not possible to obtain bacteria directly from the wounds of these patients. Patient 3 (female, age 53) suffered 35% flame burns from a paraffin heater to the legs, trunk and arms which subsequently became infected with *P. aeruginosa*, serotype 0:11 (PAJB). Patient 4 (male, age 60) received 30% burns to his left leg which became infected with *P. aeruginosa*, serotype 0:6 (PASN).

OMPs of the isolates cultivated under iron-sufficient and iron-depleted conditions (lanes 1 and 2 respectively) were transferred to NC paper and probed with serum obtained 1 and 3 weeks (patient 3), and 1 and 2 weeks (patient 4) after first signs of infection. With patient 3 (Fig. 5.8) it can be seen that the IgM response was weak (5.8, ii), but that IgG (5.8, iii) and IgA (5.8, iv) antibodies against IRMPs, D, E, G and H were evident. The reaction to protein F was initially faint but appeared to increase by the third week.

During the course of this study there were an increasing number of reports which suggested that LPS may comigrate with OMPs following separation by SDS-PAGE and remain associated with the proteins after electrophoretic transfer to NC paper (Poxton *et al*, 1985; Lam *et al*, 1987). This inferred that the bands seen on immunoblotting may represent the binding of anti-LPS antibodies to the contaminating LPS rather than to the proteins as originally presumed (previously it was considered that LPS may pass through the NC paper at the high voltages used during transfer (Sturm

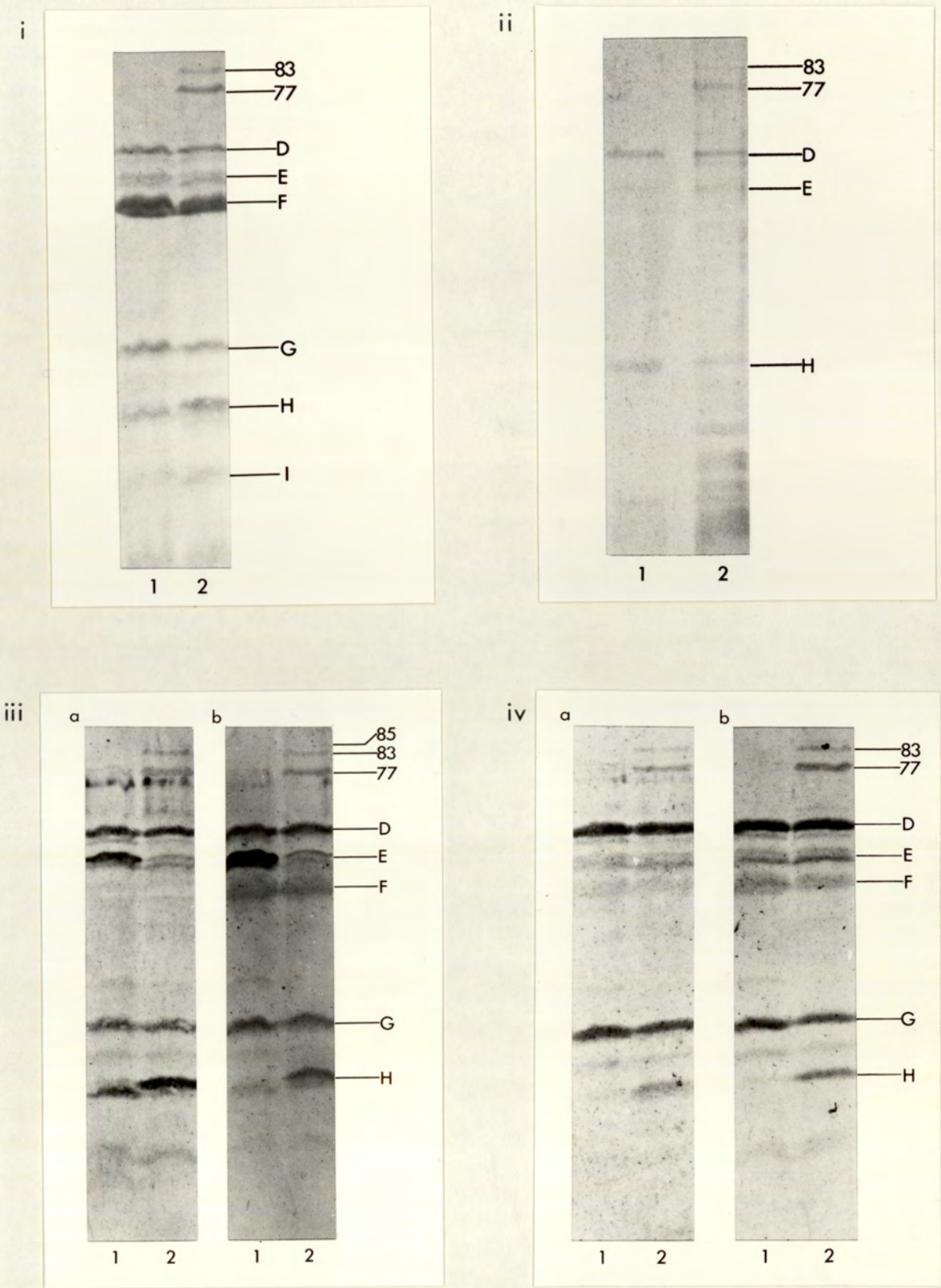


Fig. 5.8, i. OMPs of *P. aeruginosa* PAJB isolated from patient 3 and cultivated in TSB-Fe (lane 2) and TSB (lane 1). OMPs separated by SDS-PAGE, electrophoretically transferred to NC paper and stained with amido black 1%

Fig. 5.8, ii, iii and iv. Immunoblots of the OMPs described in Fig. 5.8, i, probed with serum from patient 3, followed by peroxidase-labelled anti-human IgM (ii), IgG (iii) and IgA (iv).

Serum obtained 1 and 3 weeks after onset of infection (a and b respectively). Molecular weights in kilodaltons.

et al, 1984; Shand, 1985)). To clarify this, anti-LPS antibodies in patient serum were removed by immunoprecipitation with purified LPS as described in section 2.7.4. Probing of LPS immunoblots (proteinase-K digests of OMs separated by SDS-PAGE and transferred to NC paper) with the untreated serum resulted in a weak reaction with the LPS ladder, largely to high molecular weight molecules (Fig. 5.9, lane 1), but after absorption of the serum with LPS, the ladder pattern was no longer visible (lane 2). This demonstrated that the procedure had reduced the titre of LPS antibodies in the serum to a level which no longer reacted significantly with LPS on the immunoblot. The same 2 sera were then used to probe replicate OMP immunoblots of *P. aeruginosa* PAJB cultivated in TSB-Fe (Fig. 5.9, lanes 3 and 4 respectively). There appeared to be little difference in the pattern or intensity of response between either sera, suggesting that the contribution of contaminating LPS to the bands observed was minimal. This work was carried out using serum from patient 3. Unfortunately, when the possibility of LPS contamination was realized, the serum from patients 1 and 2 had been used up, so repeating the immunoblots with LPS-absorbed serum was not possible. However, the above results suggested that the response observed with previous immunoblots largely represented a reaction to OMPs and not to LPS.

Serum from patient 4 was received at a later stage and the immunoblots shown in Fig. 5.10 were all performed with LPS-absorbed serum. In this patient, the infection had elicited IgM antibodies to IRMPs (lane 2), proteins D and E after 1 week, and to F after the second week (Fig. 5.10, ii). The pattern was similar with IgA antibodies (Fig. 5.10, iv) except an additional reaction to protein H was noted. The IgG response was much weaker and was mainly to the IRMPs and protein E.

Sera from all these patients were used to probe LPS immunoblots. In no patient was there a strong recognition of the antigen using this technique,

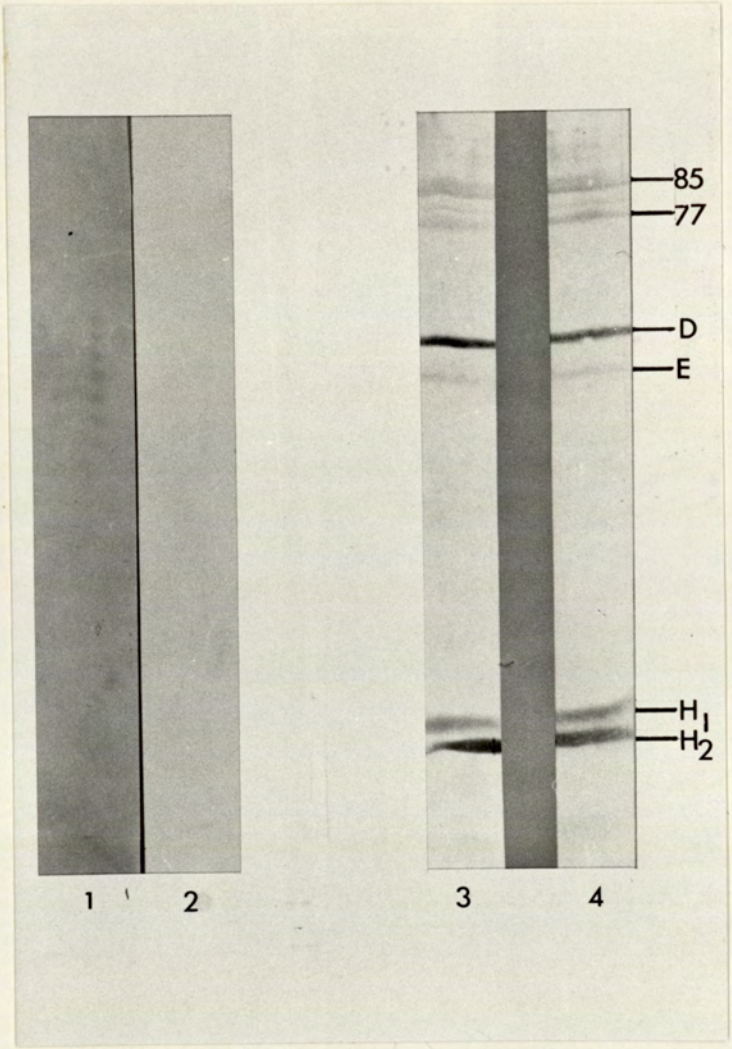


Fig. 5.9. Immunoblot of separated LPS (lanes 1 and 2) and OMPs (lanes 3 and 4) of *P. aeruginosa* PAJB, probed with untreated serum (lanes 1 and 3) and LPS-absorbed serum (lanes 2 and 4) from patient 3, followed by peroxidase-labelled anti-human IgG.

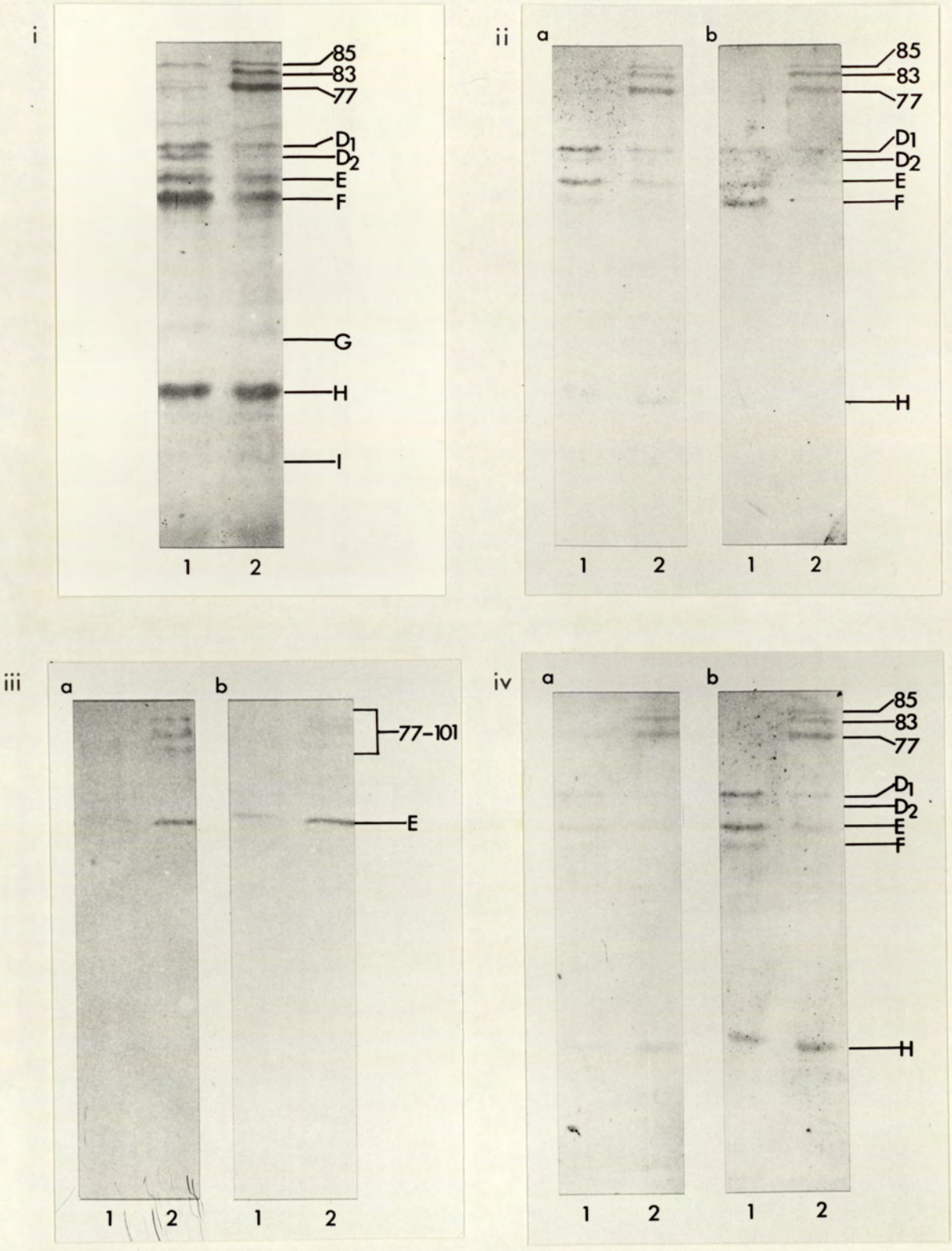


Fig. 5.10, i. OMPs of *P. aeruginosa* PASN isolated from patient 4 and cultivated in TSB-Fe (lane 2) and TSB (lane 1). OMPs separated by SDS-PAGE, electrophoretically transferred to NC paper and stained with amido black 1%

Fig. 5.10, ii, iii and iv. Immunoblot of the OMPs described in Fig 5.10, i, probed with serum from patient 4 followed by peroxidase-labelled anti-human IgM (ii), IgG (iii) and IgA (iv).

Serum obtained 1 and 2 weeks after onset of infection (a and b respectively). Molecular weights in kilodaltons.

the result for patient 3 (Fig. 5.9, lane 1) where LPS molecules with 'O'-polysaccharide side chains were faintly detected, being typical of patients 2 and 4. No detectable reaction was observed with patient 1.

5.2.2. Cross-reaction of patient antibodies with OM antigens of *P. aeruginosa* serotypes.

To determine the degree of cross-reactivity of the antibodies elicited by *P. aeruginosa* infection with the 17 IATS serotypes of the organism (Bergan, 1975), the separated OMPs of a representative of each serotype, shown in section 4.2 (Fig. 4.4) were transferred to NC paper and probed with serum from patient 1 (Fig. 5.11). IgG antibodies reacted with IRMPs of all serotypes except 0:2 and 0:14 where only faint bands were visible. Similarly, proteins D and E of all serotypes except 0:2 were recognized along with protein H of all except serotypes 0:1, 0:6, 0:12 and 0:13. There was no recognition of proteins F, G or I of any serotype with this sera obtained at an early stage of the infection.

The cross-reactivity of antibodies to IRMPs with all but 2 serotypes indicates considerable antigenic conservation of these proteins. Similar conservation was displayed by proteins D and E, and to a lesser degree, protein H. The apparent lack of reaction to protein H of certain serotypes may correlate with reduced expression of this protein in the OMs of those serotypes (Fig. 5.4). Mutharia *et al* (1982) using sera raised against OMs of *P. aeruginosa* cultivated in complex media not depleted of iron, demonstrated that proteins E, F, H_2 and I were antigenically related in all serotype strains.

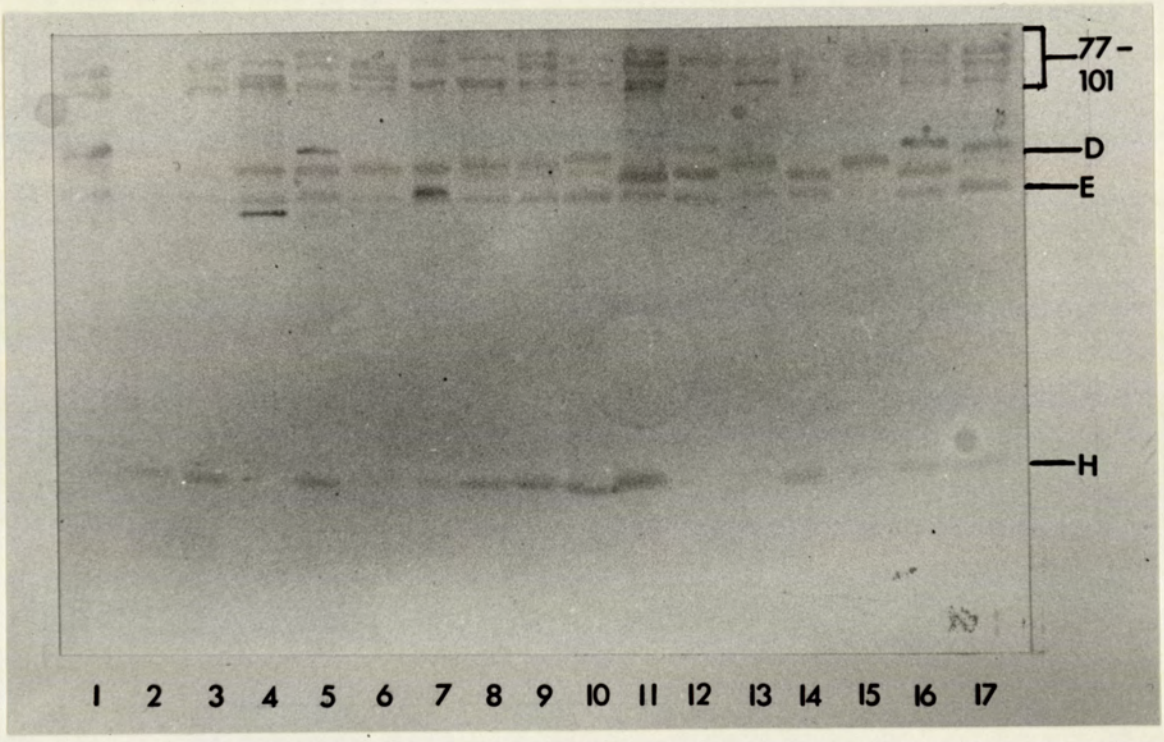


Fig. 5.11. Immunoblot of the OMPs from a representative strain of each of the 17 IATS serotypes of *P. aeruginosa* (lanes 1 to 17) as shown in Fig. 4.4. OMPs transferred to NC paper and probed with serum from patient 1 followed by peroxidase-labelled anti-human IgG. Molecular weights in kilodaltons.

5.2.3. Cross-reaction of patient antibodies with *P. aeruginosa* and other Gram-negative bacteria.

Pooled sera from 6 volunteers (4 female, 2 male; age range 35-70) with no history of *Pseudomonas* infection were used to investigate the antibody response to OM antigens of a number of Gram-negative bacteria. OMs from each strain, cultivated in TSB-Fe, were separated by SDS-PAGE and transferred to NC paper. Fig. 5.12, i, shows an amido black stain of the transferred proteins which represent *Serratia marcescens* (lane 1), *Escherichia coli* (lane 2), *Klebsiella pneumoniae* (lane 3), *Proteus mirabilis* (lane 4) and *P. aeruginosa* (lane 5). The predominant proteins in the OM of the 4 *Enterobacteriaceae* (lanes 1 to 4) were the porins with an approximate molecular weight of 40K. In each strain a number of high molecular weight (70 to 100K) iron-regulated proteins had been induced. On probing a replicate immunoblot with the pooled volunteer sera a reaction of moderate intensity with the porin proteins of the *Enterobacteriaceae* (Fig. 5.12, ii, lanes 1 to 4) was observed along with a reaction to an IRMP of *E. coli* and *P. mirabilis*. There was no discernible recognition of *P. aeruginosa* antigens (lane 5). The 4 *Enterobacteriaceae* are common residents of the gastrointestinal tract hence, a low titre of antibodies to these bacteria is not surprising. Indigenous live bacteria or bacterial antigens can penetrate the gastrointestinal epithelial barrier by a process of translocation (Berg, 1983) and can stimulate antigen-sensitive cells in the Peyer's patches, lamina propria and mesenteric lymph nodes. The humoral immune system thus becomes primed and low levels of antibodies to the resident bacteria are produced (Berg, 1985). Cross-reactivity between antigens of members of the *Enterobacteriaceae* has been demonstrated by crossed immunoelectrophoresis (Hofstra *et al*, 1980), and Chart and Griffiths (1985) showed that antibodies to the *E. coli* 0111 enterobactin receptor also recognized IRMPs

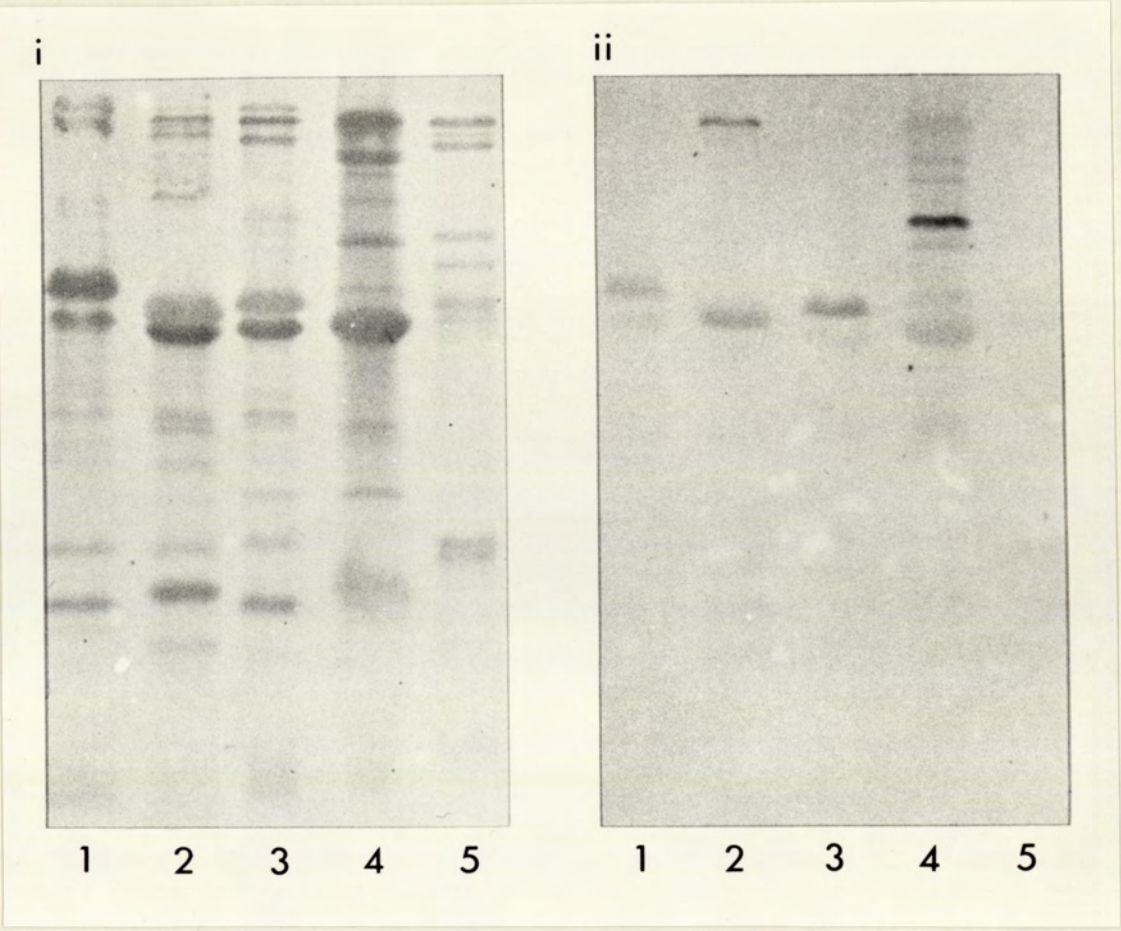


Fig. 5.12,i. OMPs of *S. marcescens* (lane 1), *E. coli* (lane 2), *K. pneumoniae* (lane 3), *P. mirabilis* (lane 4) and *P. aeruginosa* (lane 5) cultivated in TSB-Fe. OMPs separated by SDS-PAGE, electrophoretically transferred to NC paper and stained with amido black 1% w/v.

Fig. 5.12, ii. Immunoblot of the OMPs described in Fig. 5.12, i probed with pooled serum from 6 volunteers (age range 35-70) with no history of *P. aeruginosa* infection, followed by peroxidase-labelled anti-human IgG.

of *Salmonella typhimurium* and *K. pneumoniae*. Hence, the bands observed in Fig. 5.12, ii, may reflect such cross-reactions. However, there appeared to be little cross-reactivity with *P. aeruginosa* antigens, in accordance with the findings of Shand *et al* (1985). In addition, it seems probable that among the general population titres of antibodies to *P. aeruginosa* are comparatively low, only those individuals who are colonized or have had an infection with the bacteria carrying antibodies to specific *P. aeruginosa* OM components. Shand *et al* (1985) made similar observations whilst Bodey *et al* (1983) reported that only 4 to 12% of the normal population are faecal carriers of the organism. Furthermore, Young *et al* (1970) demonstrated that of 50 control subjects and 47 patients recovering from bacteraemia caused by other Gram-negative bacteria, none had precipitating antibodies to *P. aeruginosa* antigens.

When serum (Fig. 5.12, i) and tissue fluid (5.12, ii) from the burn patients described were used to probe similar immunoblots, recognition of the *Enterobacteriaceae* OM antigens was again observed (lanes 1 to 4). In both cases there was a marked reaction with an *E. coli* antigen of molecular weight approximately 81K which may correspond to the ferric enterobactin receptor described by Griffiths *et al* (1985). In addition IRMPs, proteins D, E and H of *P. aeruginosa* (lane 5) were detected. These results, taken as a whole, indicate the specificity of the response to *P. aeruginosa* antigens.

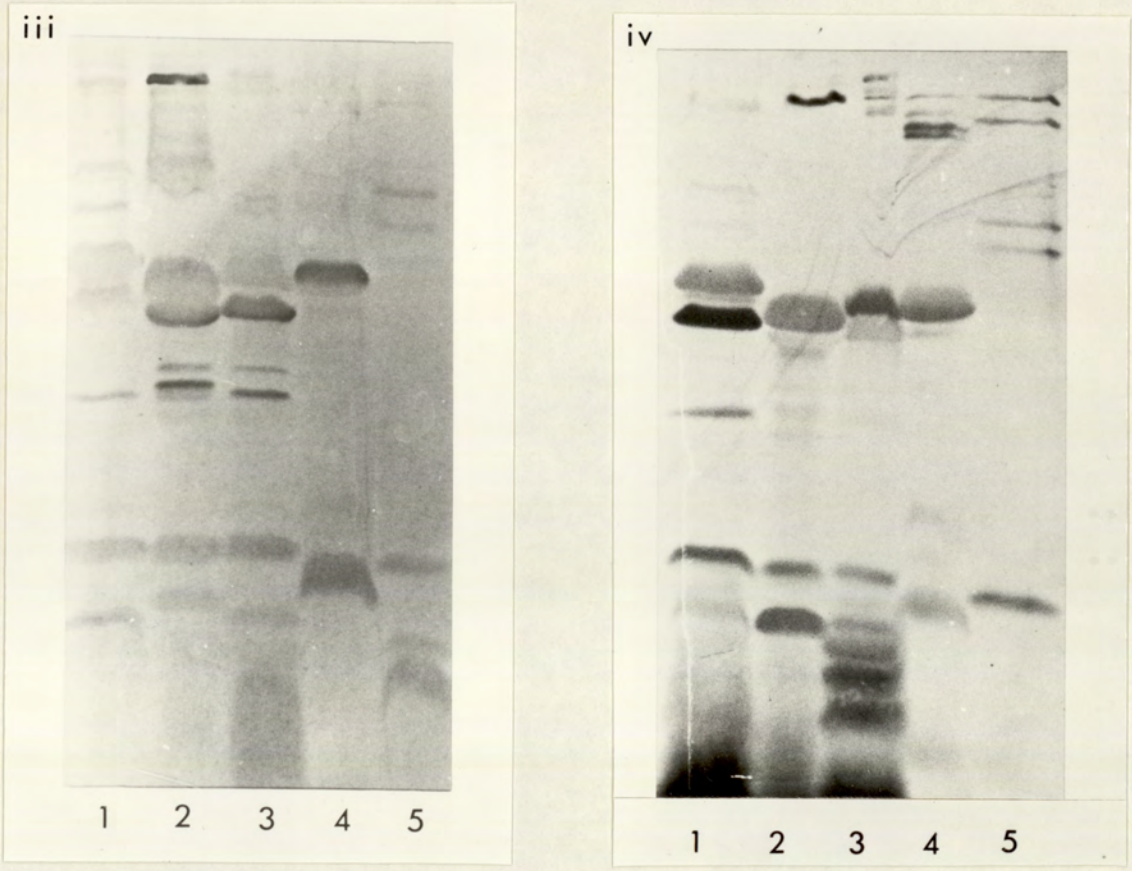


Fig. 5.12, iii and iv. Immunoblot of the OMPs described in Fig. 5.12, i, probed with serum (iii) and wound tissue fluid (iv) from burn patients infected with *P. aeruginosa*, followed by peroxidase-labelled anti-human IgG.

5.3. Discussion

Infection remains the major cause of morbidity and mortality in the thermally-injured patient (Pruitt *et al*, 1983; Kagan *et al*, 1985) and *P. aeruginosa* has emerged as a predominant member of the burn wound flora (McManus *et al*, 1985). Its ability to proliferate in the necrotic burn eschar and invade underlying viable tissue has largely determined its prevalence as a pathogen in such patients (Pruitt *et al*, 1983).

The data presented in this study indicates that the OMs of *P. aeruginosa* growing *in vivo* in burn wounds possessed markedly different characteristics to those of the same isolate grown in conventional laboratory media. The most notable variation in OMP profiles was the expression of a group of high molecular weight (77-101K) proteins in the *in vivo* bacteria which were not expressed by the isolate cultivated in complex media, but were evident following growth in the same media depleted of iron. These iron-regulated membrane proteins (IRMPs) provide direct evidence that *P. aeruginosa* grew under iron-restricted conditions in the burn wounds of the 2 patients described. Similar findings have been reported for bacteria obtained directly from human CF lung infection (Brown *et al*, 1984) or without subculture from human urine (Lam *et al*, 1984; Shand *et al*, 1985). The other minor variations observed indicate that additional differences between the *in vivo* and *in vitro* environment are important in determining bacterial surface structures. Sciortino and Finkelstein (1983) discovered some unique proteins in the OMs of *Vibrio cholerae* recovered directly from *in vivo* which were not present in iron-depleted cells grown *in vitro* and Griffiths *et al* (1983) reported non-iron related changes in the OMPs of *E. coli* harvested from the peritoneum of guinea-pigs.

Growth *in vivo* also resulted in altered LPS profiles to varying degrees when compared to corresponding *in vitro* grown bacteria. Differing

LPS migration patterns following SDS-PAGE may reflect biochemical variations in the LPS molecules (Hithcock and Brown, 1983) with consequent changes in molecular weight, or altered hydrophobicity or negativity (Kuzio and Kropinski, 1983; Pluschke *et al*, 1986). In both patients the LPS of *in vivo* bacteria contained a proportion of material that separated into bands of higher molecular weight compared to LPS of cells grown in TSB or TSB-Fe. Such material may represent LPS molecules with longer 'O' antigen side chains. Jessop and Lambert (1986) observed similar effects with LPS from *S. marcescens* grown in horse serum and TSB and suggested that differences in growth rate in the 2 environments may have accounted for this. Dodds *et al* (1987) also linked growth rate with LPS composition and demonstrated that the predominant form of LPS produced by *E. coli* at a higher growth rate was of low molecular weight.

Immunoblotting was used to determine the recognition of *P. aeruginosa* OM antigens following burn wound infection. Control experiments indicated minimal cross-reactivity between OMPs of *P. aeruginosa* and members of the *Enterobacteriaceae*, and, therefore, that any immune response was specific for *P. aeruginosa*. Further studies also demonstrated that the results obtained by immunoblotting most likely represented an antibody response to the proteins and not to contaminating LPS on the blot. Immunoglobulins of all 3 subclasses targeted against the IRMPs were detected in serum from 4 burn patients. Such antibodies were present 1 week after onset of infection. In addition, the antibodies elicited reacted with IRMPs expressed by representatives of 15 out of the 17 IATS serotypes providing further evidence that they were binding to antigenically-conserved proteins and not to serotype-specific LPS. The protective nature of antibodies to *P. aeruginosa* IRMPs is not known but, recently, Bolin and Jensen (1987) demonstrated that antibodies against *E. coli* IRMPs protected turkeys from septicaemia due to the same organism. It is possible that such antibodies

prevent uptake of iron-siderophore complexes so inhibiting vital metabolic reactions dependent on iron.

The ability of these proteins to elicit early antibody formation indicate that they are accessible to the immune system, either by virtue of their surface-location, or following phagocytic processing. Sub-MIC levels of antibiotics may also affect envelope properties of bacteria such that OMPs become surface-exposed and thus available to interact with antibodies directed against them (Kadurugamuwa *et al*, 1985b). The high molecular weight IRMPs of several Gram-negative microorganisms, including *E. coli* (Booth, 1980; Griffiths *et al*, 1983) and *S. marcescens* (Jessop, 1986), were shown to be exposed on the bacterial surface using I-125 lactoperoxidase labelling. However, as yet there is no information concerning the location of *P. aeruginosa* IRMPs.

Proteins D, E and H were also detected by patient sera, and in 3 cases were recognized 1 week after onset of infection. Lambert and Booth (1982) showed proteins D_1 and/or D_2 to be surface-localized. D_1 is believed to form a glucose-inducible pore in the OM (Hancock and Carey, 1980) and, as such, it is likely that at least part of the polypeptide chain would be exposed on the surface. However, there was no evidence for the labelling of E, H_1/H_2 or I which may infer that either they are more deeply embedded in the membrane or else do not bear tyrosine or histidine residues on surface regions of their polypeptides to which I-125 lactoperoxidase can bind. Monoclonal antibodies with indirect immunofluorescent techniques can also be used to determine surface localization of antigens. Mutharia and Hancock (1983) using monoclonal antibodies to protein H_2 found no fluorescence with intact wild type cells cultivated in complex media but did demonstrate a reaction with a rough LPS-deficient mutant. Hence, they suggested that only strains lacking LPS 'O' side chains have protein H_2 as a surface antigen. H_1 and H_2 were not resolved under the electrophoretic conditions employed

in this study, hence it was not possible to distinguish which of the 2 proteins had elicited antibody formation. The apparent lack of recognition of the 'H' protein in the *in vivo* grown cells is surprising. It is not known whether this was due to reduced expression of the protein or loss of antigenic sites on the protein following extraction from wound tissue.

The results also indicated that the porin protein F was not initially detected by serum antibodies, but that in some cases, a response to this protein was observed after 2 or 3 weeks. Mutharia *et al* (1982) reported a low transfer efficiency for this protein on immunoblotting. However, amido black staining of replicate immunoblots after electrophoretic transfer implyied that sufficient protein F was present on the NC paper to be detected by the stain and, hence, also by serum antibodies. The late response to protein F was further demonstrated in a rabbit peritoneal model of infection (section 6.5), and may be interpreted as suggesting the protein is less antigenic than other OMPs or less accessible to the immune system. However, Lambert and Booth (1982) and Mutharia and Hancock (1983) using different techniques both demonstrated that the porin is located on the cell surface. A possible explanation is that certain epitopes on the protein which may have induced early antibody formation are lost on boiling with sample buffer containing 2-mercaptoethanol prior to SDS-PAGE. Mutharia and Hancock (1985) characterized 2 surface-located antigenic sites on protein F using 4 monoclonal antibodies. Of these, 3 reacted with the native protein and trypsin- and papain-derived fragments, but not with 2-mercaptoethanol-reduced protein. The 4th recognized epitopes which were not destroyed by 2-mercaptoethanol but were lost after papain or trypsin treatment. Such 2-mercaptoethanol-resistant epitopes may elicit a later immune response which can be visualized on immunoblotting. Recognition of protein F by serum from patients with urinary tract infection was demonstrated using immunoblotting (Shand, 1985; Shand *et al*, 1985), but it is not known at what stage in the

infection the serum was obtained. Additionally, serum from CF patients with chronic lung infection strongly detects this antigen (Anwar *et al*, 1984) but such patients are exposed to *P. aeruginosa* for several years so enabling many epitopes on the protein to stimulate antibody formation. Hence, the poor reaction to protein F in early serum samples in this study may be an artefact of the technique, or may reflect the true response to this protein following onset of burn wound infection. The protective capacity of purified protein F (Gilleland *et al*, 1984; Matthews-Greer and Gilleland, 1987) or monoclonal antibodies to the protein (Hancock *et al*, 1985) has already been clearly established (see section 1.5.2) the antigen eliciting high titres of IgG antibodies.

Immunoblotting with serum from patient 1 demonstrated the recognition of flagella protein shown in other studies to be highly immunogenic (Mutharia *et al*, 1982; Anwar *et al*, 1984; Shand , 1985). The antigen was not evident in the OMs of *P. aeruginosa* strains from other patients but this is probably because anchoring of flagella in the OM may vary from strain to strain. As such, flagella from some strains may be more easily sheared and lost during OM preparation. Motility is thought to be an important virulence factor in invasive wound sepsis (McManus *et al*, 1980; Holder, 1985) and it has been suggested that a functioning flagellum is needed for successful invasion of surrounding tissue by the infecting bacteria (Montie *et al*, 1982b). The efficacy of flagella antigens as vaccine candidates has been discussed in section 1.5.2.

The presence of antibodies locally in the wound was investigated in 2 patients and a pattern of response similar to that seen with serum was observed. Following colonization, bacteria on the surface of the wound penetrate the eschar to varying degrees depending on their invasive capacity and the local host defence capabilities (Pruitt and McManus, 1984). *P. aeruginosa* produces proteases and other extracellular factors which lyse

the denatured collagen of the eschar so facilitating invasion of underlying viable tissue. Local immune defences are, therefore, critical in halting the development of invasive wound sepsis (Deitch *et al*, 1985). The 2 patients in question did not become septicaemic following colonization with *P. aeruginosa*, and the infection was successfully cleared in both cases. The presence of antibodies in the viscinity of the burn eschar against OMPs including IRMPs may have helped prevent such severe consequences.

The antibody response to *P. aeruginosa* antigens has been investigated by other researchers in different types of infection, the results not always reflecting those seen with burn patients. Hedstrom *et al* (1984) investigated the detection of OMPs of the organism by mice immunoglobulins following establishment of a subcutaneous abscess. Serum was obtained 14 days after onset of infection and was found to recognize proteins F, H_2 and I, along with an antigen of approximate molecular weight 16K which they presumed to be pilus protein. Cells used for immunoblotting in that study were not cultivated in an iron-depleted medium, hence the response to IRMPs could not be determined. It is possible that species variation may account for some of the differences in the immune response observed. Serum from patients with urinary tract infection due to *P. aeruginosa* barely detected the IRMPs, whereas concentrated urine from the same patient clearly had antibodies to these proteins (Shand, 1985). Proteins D, E, F, H and flagella antigen were additionally recognized, a response more closely resembling that of burn patients. Sera from CF patients detected IRMPs, proteins D, and H_2 and showed a particularly strong reaction with proteins F and G. In addition, a band below H_2 had stimulated a marked response, which was postulated as representing rough LPS running at the front of the gel (Anwar *et al*, 1984). Strong recognition of this antigen was not observed with the burn patients in this investigation. *P. aeruginosa* growing in the CF lung causes a chronic infection. As mentioned in section 1.3, the bacteria are

seen to grow in glycocalyx-enclosed microcolonies embedded in their own exopolysaccharide which persist despite antibiotic therapy and host defences, and which frequently release mobile swarmer cells resulting in exacerbations of pneumonia (Costerton, 1984). Therefore, over the years *P. aeruginosa* exposes many antigens to the immune system so stimulating the production of high titres of antibodies to numerous bacterial components (Høiby and Axelsen, 1973; Høiby, 1977; Lam *et al*, 1983). In contrast, *P. aeruginosa* in burn wounds produces an essentially acute and possibly life threatening infection involving largely mobile cells which are not maintained in the 'cryptic' state seen in the CF lung. These cells are highly invasive releasing toxins and other extracellular products and sometimes causing bacteraemia (Costerton *et al*, 1983). Hence, it seems that the nature (chronic/acute) and site of infection as well as the host may influence the pattern of the immune response to *P. aeruginosa* OM antigens.

The value of vaccination in chronic infections such as CF is questionable in part due to problems of damaging immune complex formation which may further complicate the clinical situation (Berdischewsky *et al*, 1980; Woods and Bryan, 1985). However, in burn patients active immunization with vaccines or passive immunotherapy may reinforce host defences to overcome the acute, invasive attack. The many different approaches to the immunotherapy of *P. aeruginosa* infection currently undergoing experimental evaluation have been described more fully in section 1.5. It only remains here to emphasize the potential of OMPs as vaccine candidates. Such antigens possess several desirable characteristics, being non-toxic, non-pyrogenic and antigenically conserved among serotype strains. Furthermore, studies have indicated that major OMPs directly interact with and mitogenically stimulate B-lymphocytes (Chen *et al*, 1980). The use of OMPs from enteric bacilli as vaccines may not be as profitable since studies have shown them to be less accessible to antibodies in wild type cells

partly due to shielding by LPS molecules (van der Ley *et al*, 1986). However, OMP vaccines for non-enteric Gram-negative bacteria including *P. aeruginosa* still hold much promise (Gilleland and Matthews-Greer, 1987). Researchers have already considered protein F in this capacity with encouraging results (Gilleland *et al*, 1984; Matthews-Greer and Gilleland, 1987). This study has identified other antigens which elicit early antibody formation during the course of burn wound infection and hence should perhaps be considered either as vaccine candidates, or for monoclonal antibody production for future passive immunotherapy.

6. Antibody response to *P. aeruginosa* growing as an adherent biofilm or as a disseminated peritonitis.

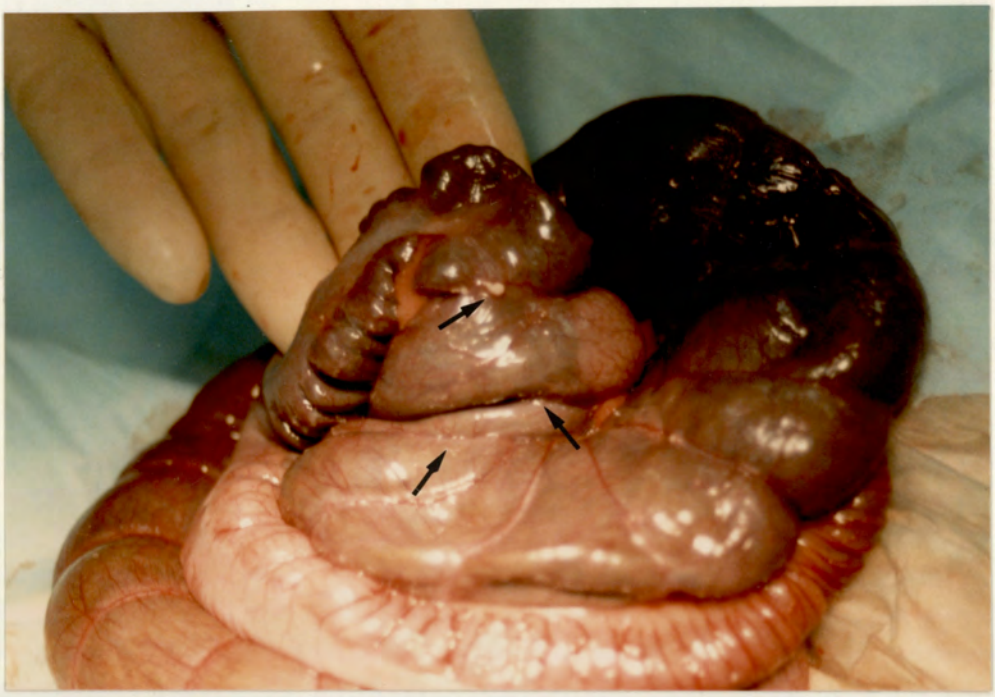
6.1. Rabbit peritoneal infection models

Two rabbit models of infection were established. The first involved a disseminated peritonitis and the second, a localized infection characterized by bacteria adhering to the surface of implanted biomaterials.

In the disseminated infection, a disc of Silastic sheeting (section 3.3) (diameter 1.5cm), following incubation in an overnight culture of *P. aeruginosa* (PAO-1) in CDM-Fe , was implanted unwashed into the rabbit peritoneum. Approximately 10^7 bacteria were associated with each disc including droplets of the culture which were carried over with the disc. 3 of 7 rabbits died 24 hours following implantation, from presumed LPS toxicity. Of the rabbits which survived, a laparotomy performed 4 days after insertion of the disc showed evidence of disseminated infection, as seen in Fig. 6.1. Beads of pus (marked with arrows) were present on tissues throughout the peritoneal cavity, distal from the disc. This indicated that at some stage planktonic bacteria had been released into the peritoneum to colonize other tissue surfaces. After 4 weeks there were no signs of infection in the peritoneal cavity, except for bacteria remaining on the disc. The number of bacteria which could be recovered from the surface of the disc by scraping with a sterile scalpel blade varied from $1x10^5$ to $5x10^7$ and, in one instance, the disc was found sealed off in a pocket of pus and white cells in the form of an abscess. As many as $4x10^8$ bacterial cells were recovered.

This model thus represented a disseminated peritonitis, which at some point involved a planktonic population of bacteria that spread to colonize tissues throughout the peritoneum. The acute disseminated phase of the

6.1, i.



6.1, ii.

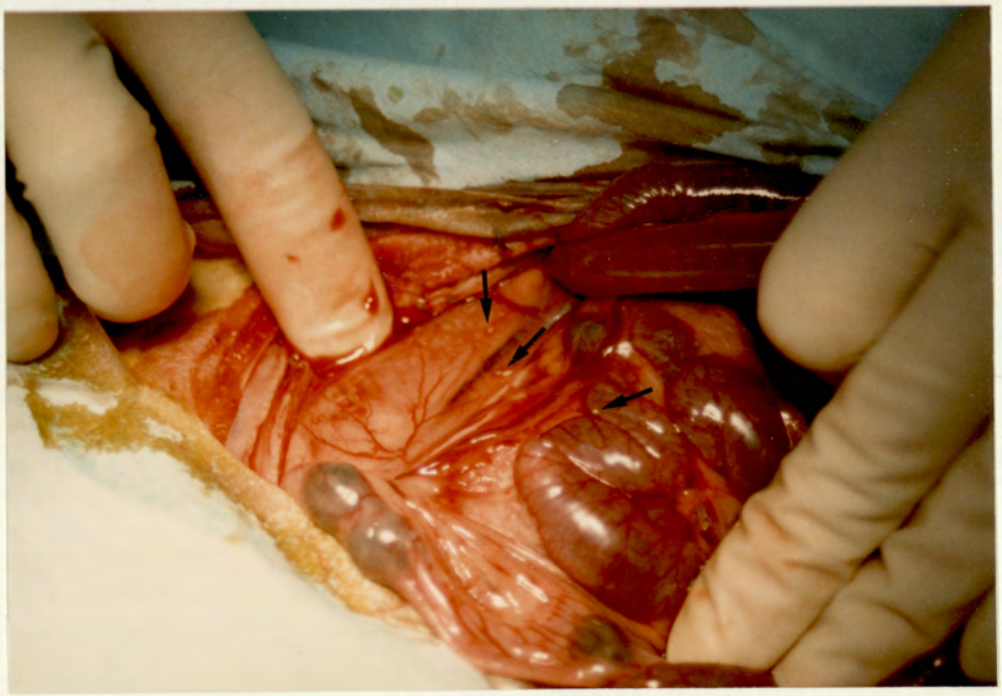


Fig. 6.1, i and ii. Results of a laparotomy performed on a rabbit with disseminated peritonitis, 4 days after onset of infection. Arrows indicate beads of pus on tissues and organs throughout the peritoneal cavity, implying spread of bacteria from the initial site of infection (1 disc of Silastic sheeting colonized with *P. aeruginosa* PAO-1).

infection was effectively cleared, but bacteria adherent on the initial disc remained.

In the biomaterial associated infection, devices as described in section 3.3.2 each consisting of 10 discs of Silastic sheeting (as used in Tenckhoff catheters (Tenckhoff and Schechter, 1968) were incubated in a dilute bacterial suspension (10^5 /ml) for 3 h at 37°C. This enabled bacteria to adhere to the surface of the discs. Two such devices with approximately 10^5 bacteria adherent on the disc surfaces were then implanted into the peritoneum. No rabbits died at any stage in the course of the study, and a laparotomy performed 4 days after implantation showed no evidence of dissemination of infection in the peritoneal cavity (Fig. 6.2). The infection remained essentially localized on the disc surfaces and after 4 weeks, the peritoneal cavity still had no signs of infection (Fig. 6.3). However, the devices were clearly heavily colonized as shown in Fig. 6.4, with large numbers of white cells associated with the discs. On dismantling both devices and scraping the disc surfaces, coupled with vortexing to dislodge the majority of cells, 10^8 to 4 x 10^9 adherent bacteria (*P. aeruginosa*) were recovered.

Figs. 6.5, i and 6.5, ii, show scanning electron micrographs of the disc surfaces after dismantling the devices. Rod-shaped bacteria were evident, often grouped in microcolonies and some partially or fully embedded in an extensively dehydrated structure of bacterial or possibly host origin. This is characteristic of glycocalyx-enclosed adherent microcolonies of bacteria (Costerton *et al*, 1985; Gristina and Costerton, 1985). The fibrous glycocalyx matrix is well-hydrated in its natural state and partially collapses during the rigorous dehydration steps used to prepare samples for scanning electron microscopy (SEM), revealing bacteria adherent to the surface beneath and partly coated with the condensed amorphous residue (Gristina and Costerton, 1985).

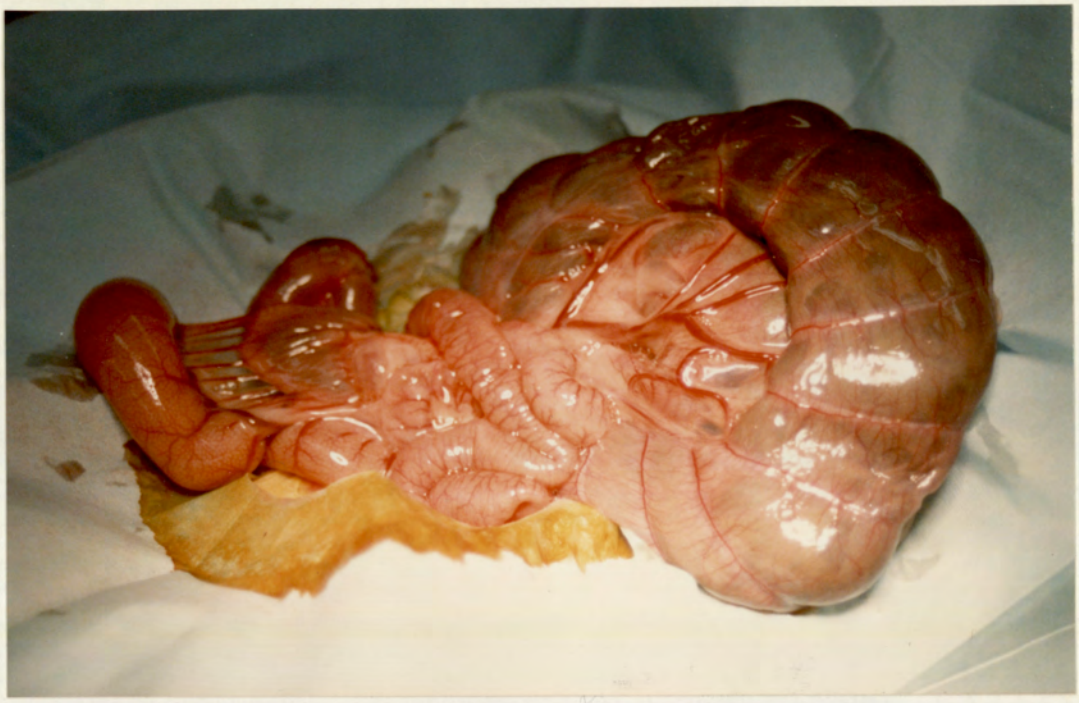


Fig. 6.2. Results of a laparotomy performed on a rabbit with biomaterial-associated infection, 4 days after implantation of the devices (section 3.3.2.). No signs of disseminated infection were apparent.

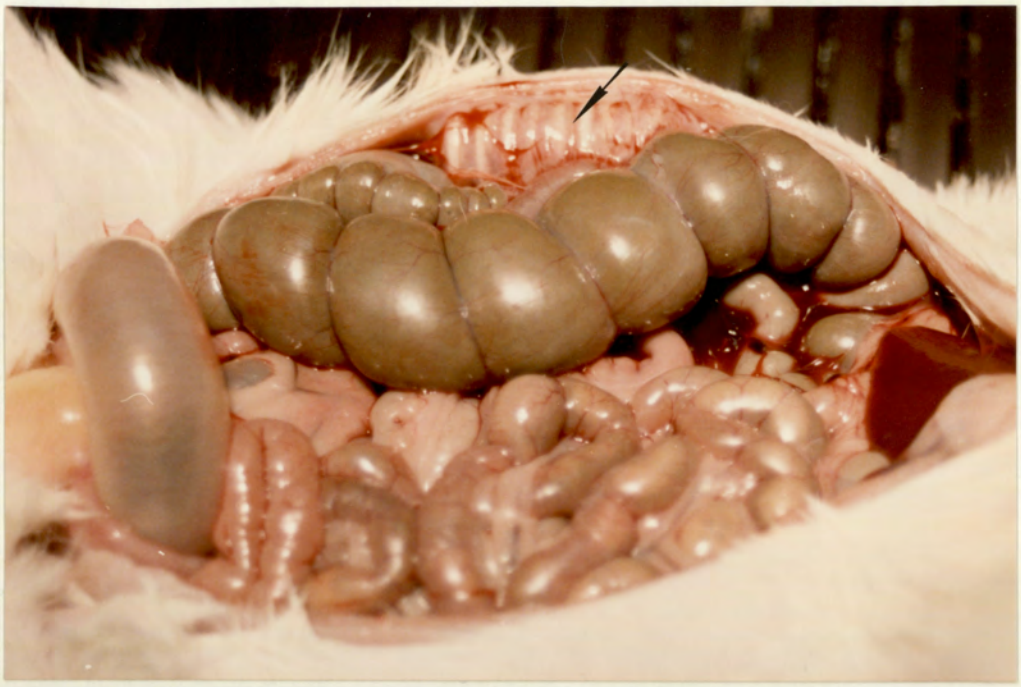


Fig. 6.3. Results of a laparotomy performed on a rabbit with biomaterial-associated infection, 4 weeks after implantation of the devices (position of 1 device marked with arrow). No signs of infection throughout the rest of the peritoneal cavity indicating that the infection had remained localized.

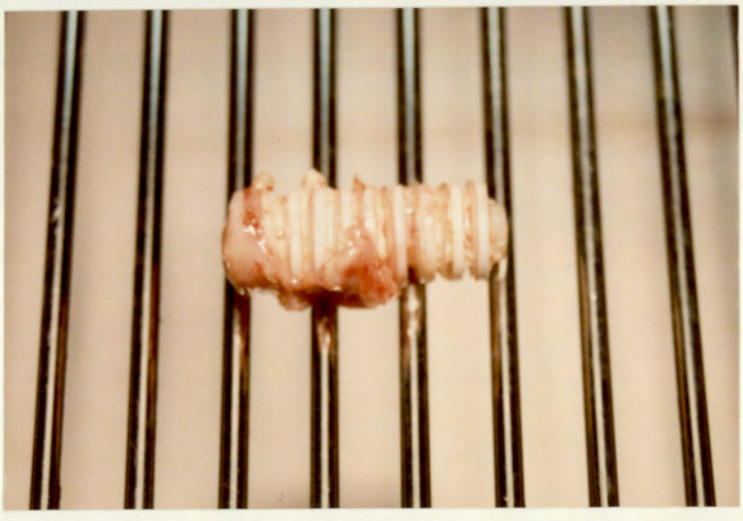


Fig. 6.4. Device removed from the peritoneal cavity 4 weeks after implantation showing heavy colonization and large numbers of white cells in the vicinity of the discs.

Transmission electron micoscopy (TEM) of ruthenium red-stained scrapings from the disc surfaces (Fig. 6.6) demonstrated aggregates of rod-shaped bacteria with a typical Gram-negative cell envelope, amongst disintegrated cellular material, possibly leucocytes. Electron-dense, partially collapsed glycocalyx material was seen surrounding the cells and scattered in the intercellular spaces. Prior to glutaraldehyde fixation, the samples were antibody-stabilized by incubation in antisera to *P. aeruginosa* PAO-1 whole cells to prevent total dehydration of the glycocalyx structure (Mackie *et al*, 1979). TEM showed that the fibrous material produced by the bacteria was only partly stabilized by this procedure, and was seen to be condensed at the cell surface. The high density of polymorphonuclear leucocytes and macrophages surrounding the bacteria possibly inhibited the penetration of antibodies and hence prevented full stabilization of the fibrillar matrix. Alternatively, the titre of antibodies in the serum was too low or the contact time with the cells insufficient.

Similar glycocalyx-enclosed bacterial biofilms have been demonstrated by SEM and TEM techniques on the surfaces of a variety of implanted prostheses. These include urinary, intravenous, intraarterial and Tenckhoff catheters (Peters *et al*, 1981; Marrie *et al*, 1983; Marrie and Costerton, 1984; Nickel *et al*, 1985a), intrauterine contraceptive devices (Marrie and Costerton, 1983a) and percutaneous sutures (Gristina *et al*, 1985).

In this model, therefore, a predominantly sessile population of bacteria was established growing as an adherent glycocalyx-enclosed microcolony on the disc surfaces. The infection remained localized with no evidence of dissemination but high numbers of bacteria (10^8 to 4×10^9) accumulated within the biofilm by week 4 and possibly earlier.

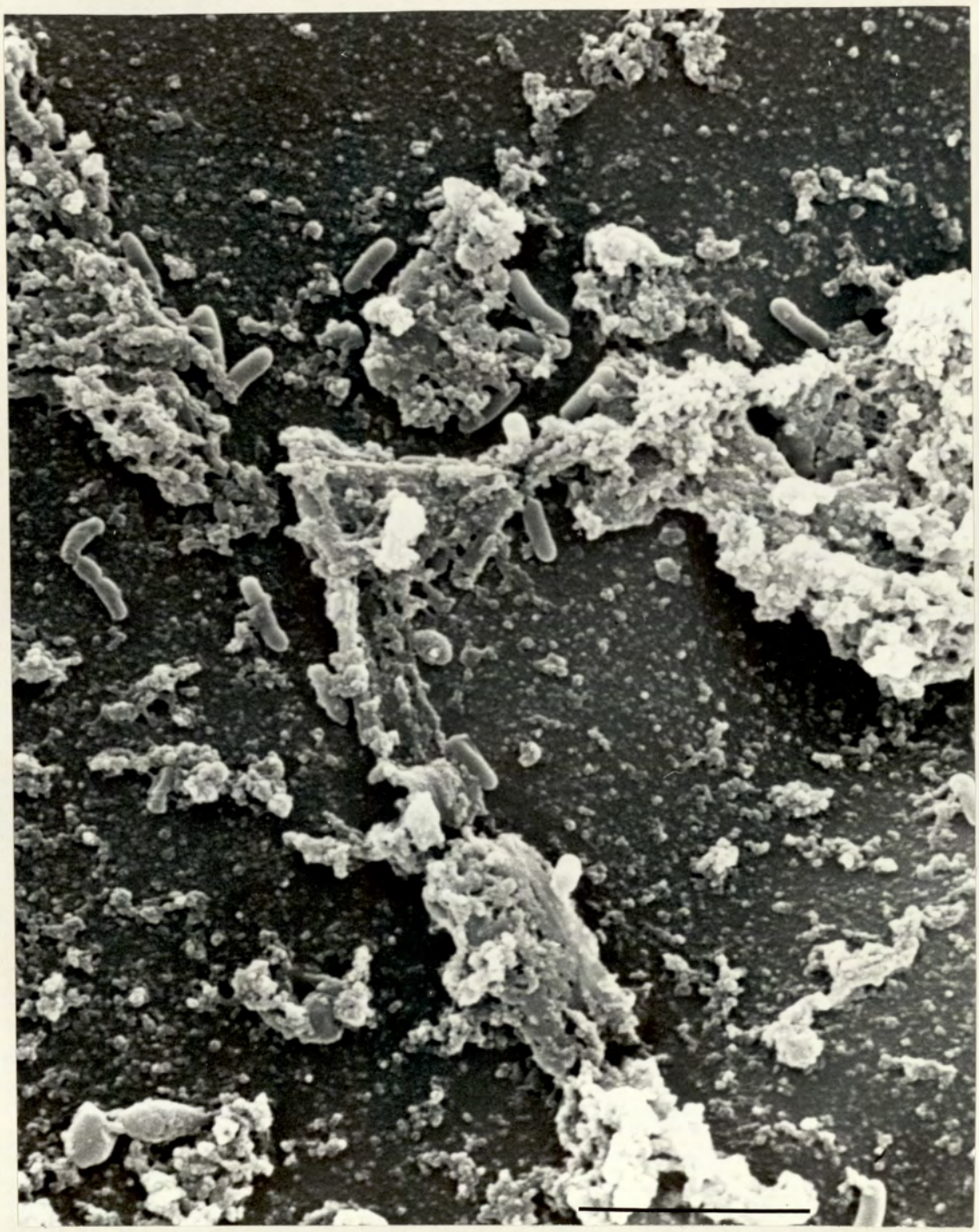


Fig. 6.5, i. Scanning electron micrograph (SEM) of the surface of a disc of Silastic sheeting, 4 weeks after implantation of the devices. Rod-shaped bacteria can be seen coated with, or partially buried within, the extensively dehydrated remains of the glycocalyx (of bacterial or possibly host origin). Bar=5μm.

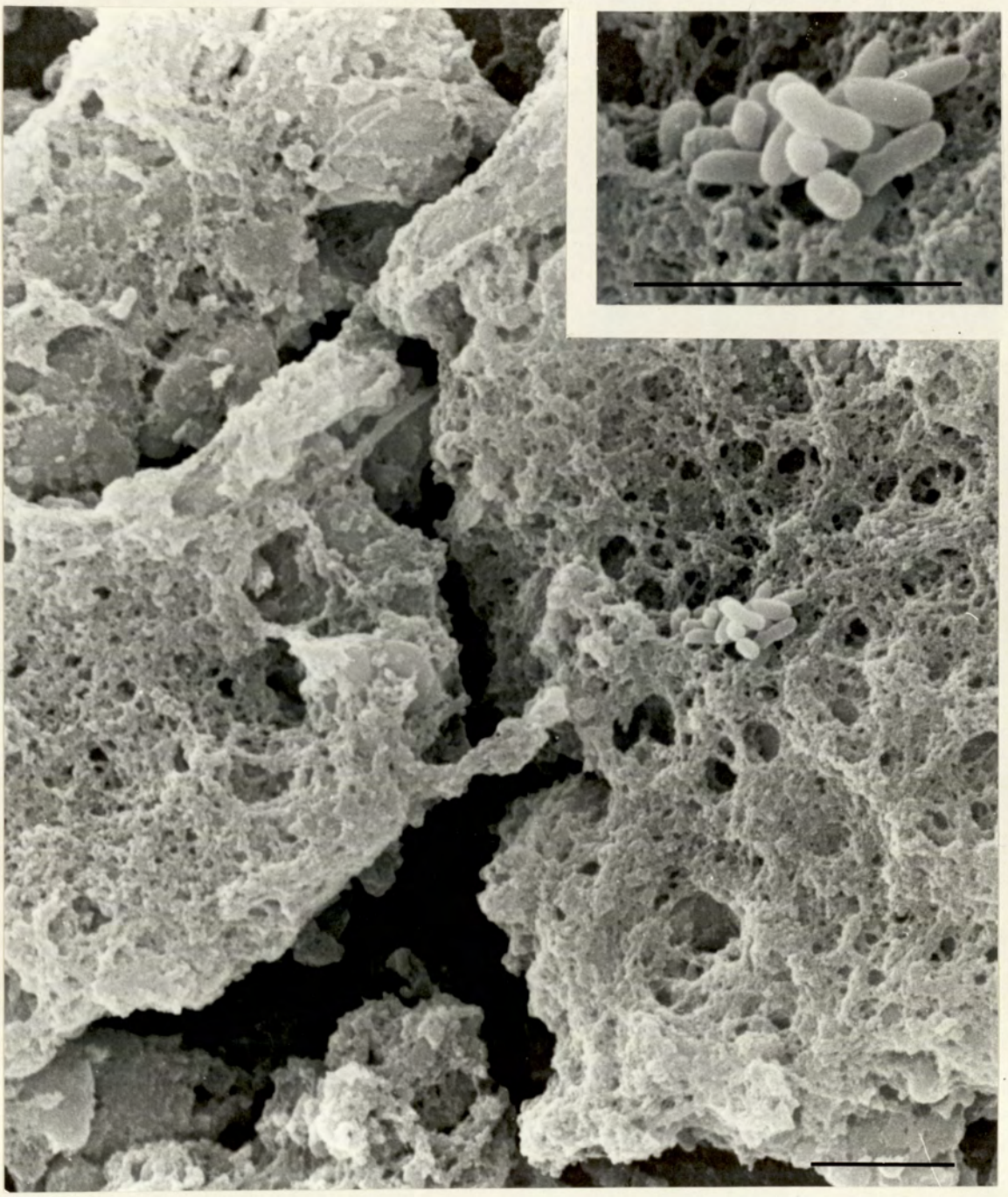


Fig. 6.5, ii. SEM as described in Fig. 6.5, i. Note the development of a discrete adherent microcolony of bacteria of a single morphotype, partly embedded in condensed, amorphous material. Inset shows the microcolony at a higher magnification. Bar=5μm.

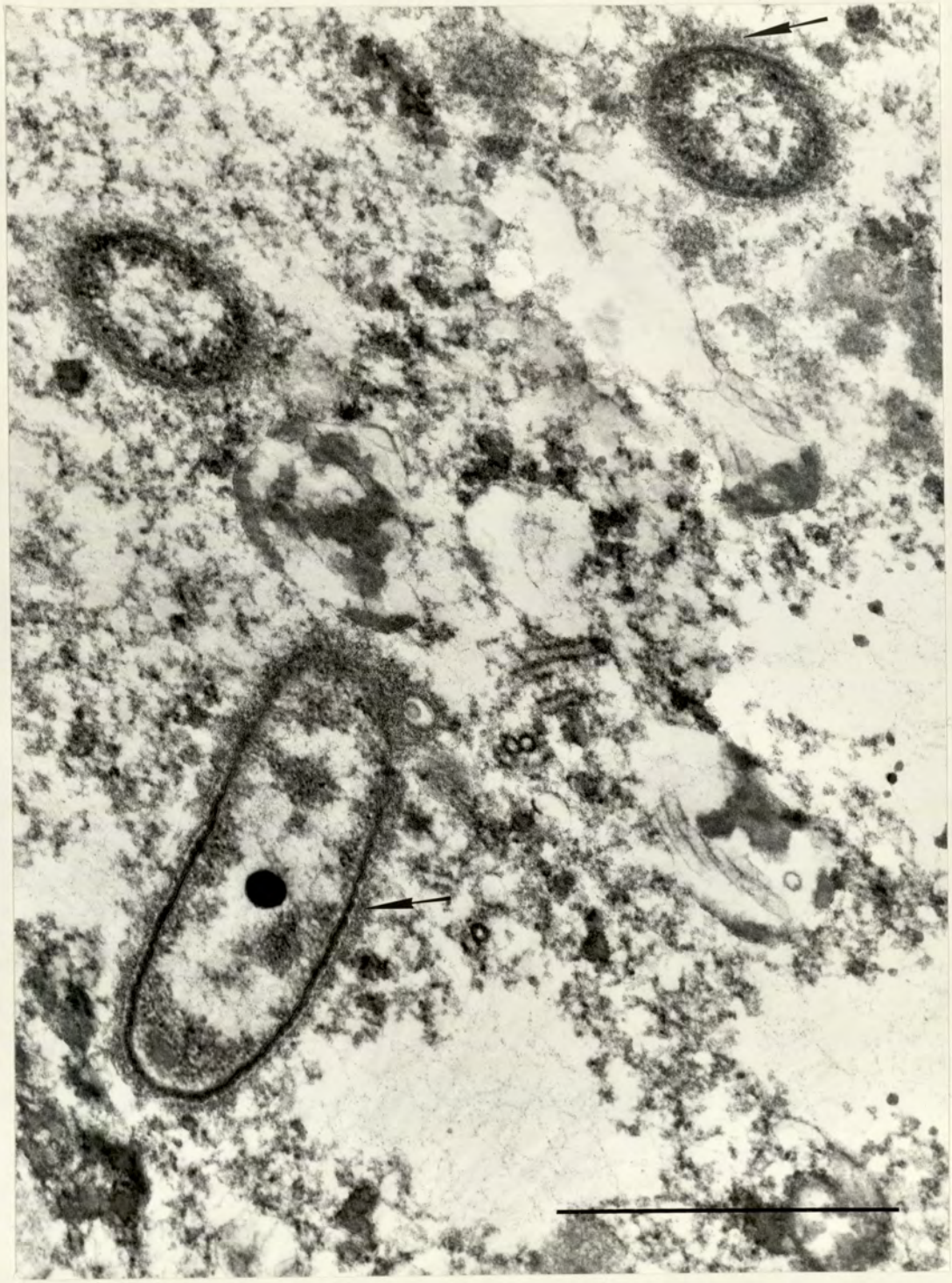


Fig. 6.6, i. Transmission electron micrograph (TEM) of a ruthenium red-stained preparation of biofilm material scraped from the surface of a disc of Silastic sheeting, 4 weeks after implantation of the devices. Preparation was incubated in specific antisera before processing. Partially-stabilized, fibrous anionic matrix can be seen surrounding the rod-shaped cells (arrows) and collapsed glycocalyx material forming coarse, electron dense aggregates is present in some areas . Bar=1μm.

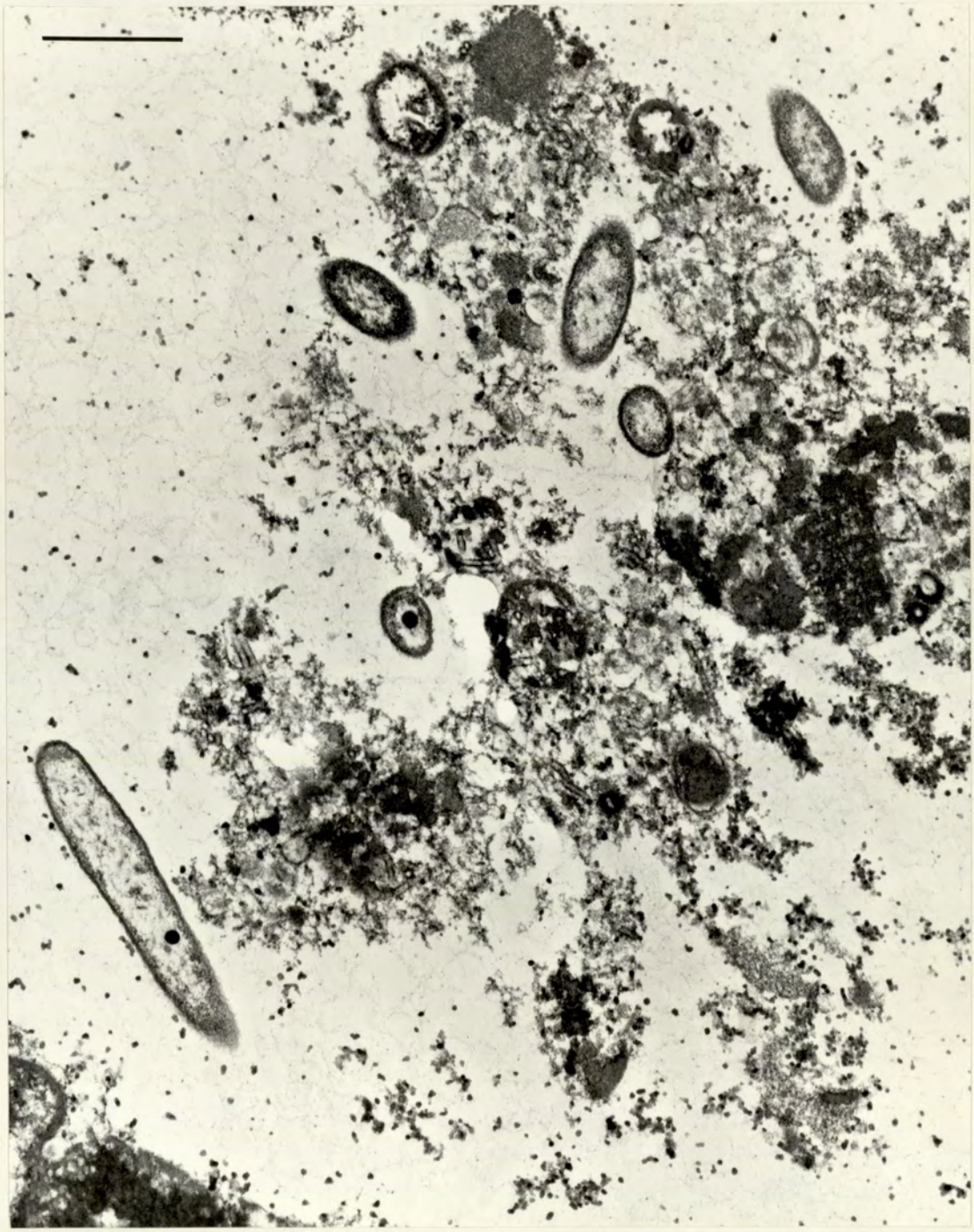


Fig. 6.6, ii. TEM as described in Fig. 6.6, i, and showing a microcolony of Gram-negative cells partly embedded in dehydration-condensed extracellular glycocalyx residue. Bar=1μm.

6.2. Sequential rabbit humoral immune response to *P. aeruginosa* antigens studied by crossed immunoelectrophoresis.

Crossed immunoelectrophoresis (CIE) was carried out as a non-denaturing method for studying the immune response to reference antigens of *P. aeruginosa* PAO-1. The antigens used in this system were prepared from whole cells of *P. aeruginosa* PAO-1 cultivated in CDM-Fe, and disrupted by French press and sonication.

When serum from a rabbit immunized with formalin-killed *P. aeruginosa* PAO-1 whole cells over a period of 8 weeks (section 2.7.1) was run against this antigen system, a complex pattern of precipitin peaks was produced (Fig. 6.7). More than 30 antigens migrating towards the anode in the first dimension were routinely observed. Although the pattern produced was not identical to that reported by Lam *et al* (1983), there were no major differences in the more prominent precipitins. This antigen system was then used as a base-line reference to investigate antibody production to particular antigens during the course of the two infection models described.

The results using serum taken sequentially from 3 rabbits with disseminated peritonitis are shown in Fig. 6.8, i, ii and iii, respectively. In all 3 rabbits, pre-infection serum (labelled 'Pre') showed no detectable response to any of the antigens. Although no antigens were recognized by one rabbit until 8 days after onset of infection (Fig. 6.8, ii), faint precipitins were visible by day 4 for the other 2 rabbits (Fig. 6.8, i and iii). In all cases, as the course of the infection was followed, a particular peak, marked with the black arrow, was seen to intensify and diminish in height as time progressed. This is indicative of a marked and increasing antibody response to that antigen. The migration pattern of this antigen resembled that of a precipitin peak described as LPS by Lam *et al* (1983). Crossed-line immunoelectrophoresis (Kroll, 1973) involving incorporation of purified LPS into the intermediate gel before electrophoresis in the second

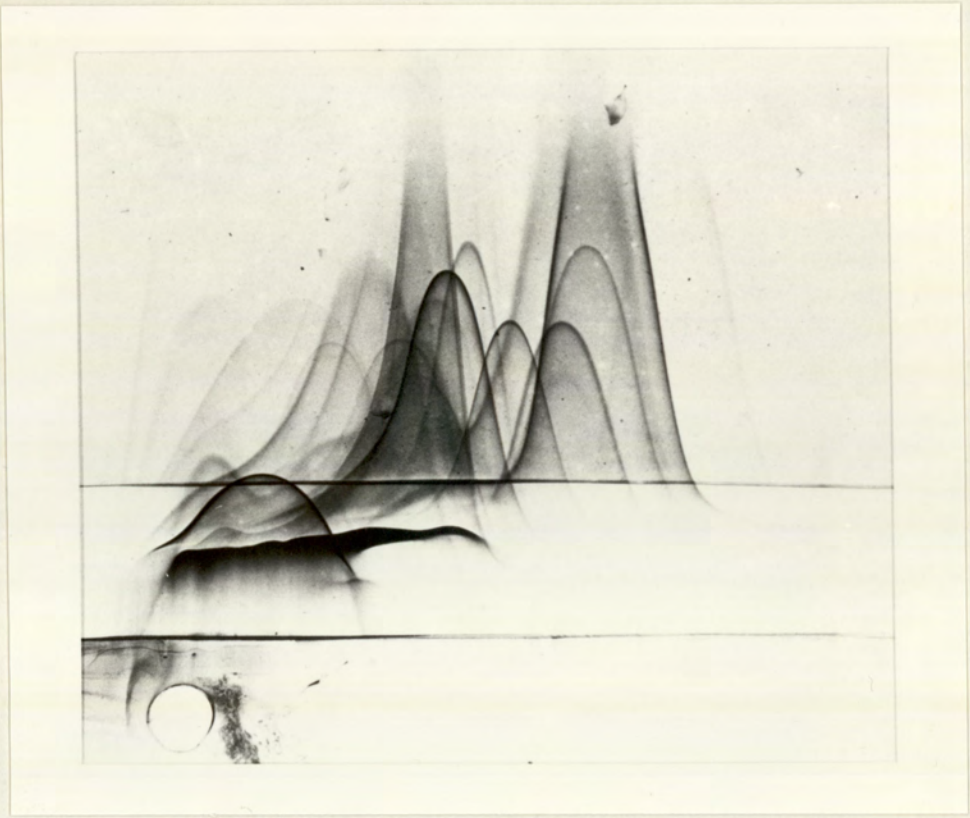


Fig. 6.7. Crossed immunoelectrophoresis of *P. aeruginosa* PAO-1 antigens (120μg protein) run against antisera from a rabbit immunized with formalin-killed *P. aeruginosa* PAO-1 whole cells. Saline was added to the intermediate gel. The anode is to the right and top of the gel and the gel was stained with Coomassie brilliant blue.

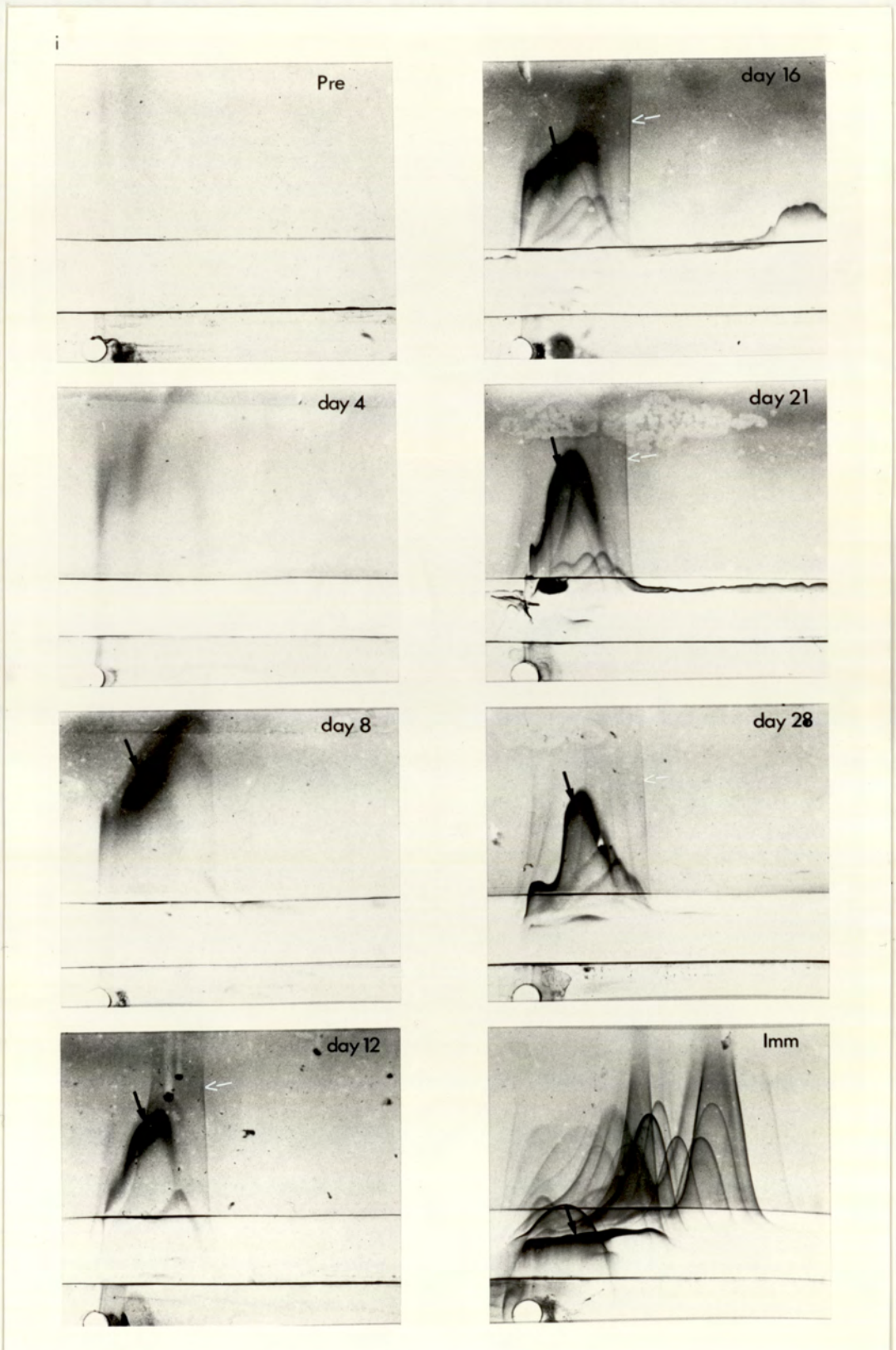


Fig. 6.8, i, ii and iii. Crossed immunoelectrophoresis of *P. aeruginosa* PAO-1 antigens (120μg protein) run against sequential serum samples obtained from 3 rabbits (6.8, i, ii and iii respectively) with disseminated peritonitis.

'Pre' refers to pre infection serum.

'Days' refer to number of days after onset of infection.

'Imm' refers to homologous rabbit antisera as described in Fig. 6.7.

Fig. 6.8, ii.

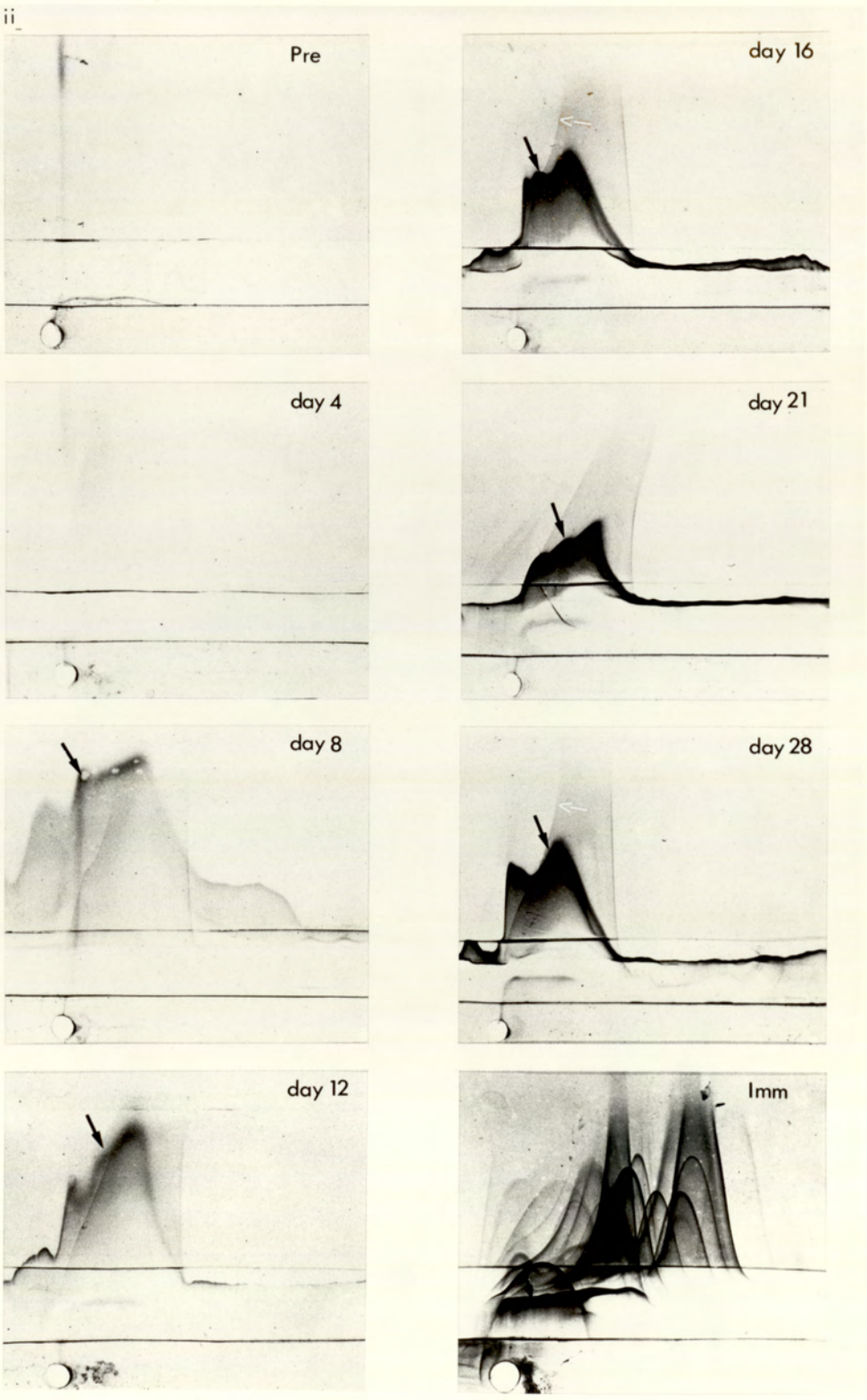
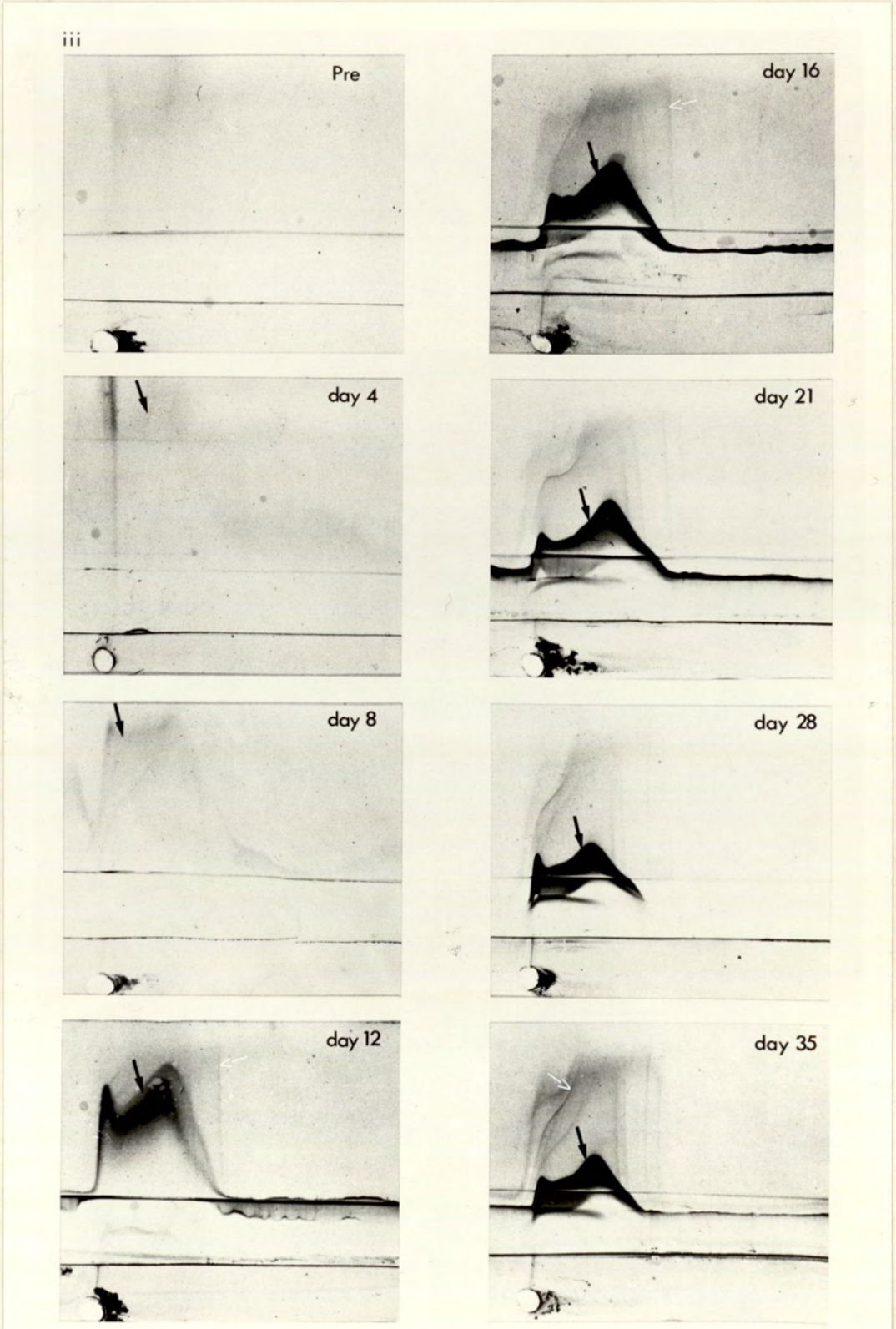


Fig. 6.8, iii.



dimension, confirmed the identity of the peak as shown in Fig. 6.9. Using immunized rabbit serum, the presence of LPS in the intermediate gel resulted in the disappearance of the peak marked with the arrow (Fig. 6.9, i), and the formation of a dark precipitin line which extended across the whole reference system (Fig. 6.9, ii). With infected rabbit serum the precipitin in question formed a high rocket peak, and inclusion of LPS produced a line of identity which was seen to fuse with this peak (Fig. 6.9, iv). The darkly staining nature of the precipitin with Coomassie brilliant blue suggests that it may be LPS or an LPS/protein complex (Lam *et al*, 1983). This antigen was detected 4 to 8 days after onset of infection and had evoked a strong antibody response in all rabbits by day 16. Additional high rocket peaks were seen developing as the infection continued, and one rabbit (Fig. 6.8, iii) demonstrated up to 7 more precipitins by day 21. Precipitins indicated with white arrows were common to most rabbits but their identity is unknown.

Similar studies were performed with serum from 4 rabbits with biomaterial-associated infection, and a different immune response was seen, as indicated in Fig. 6.10. The most notable variation was the absence of the darkly staining antigen corresponding to LPS. In 3 of 4 rabbits (Fig. 6.10, i, ii and iii), this was true even 5 weeks after implantation of the infected devices and hence at least a week after 10^8 to 4 x 10^9 bacteria were shown to be adherent on the Silastic disc surfaces. In the 3rd rabbit the devices were left in the peritoneum for a further 3 weeks and serum obtained on day 60. There was still no marked response to the LPS antigen (Fig. 6.10, iii, day 60). With the 4th rabbit the familiar LPS precipitin was seen developing but not until day 21 (Fig 6.10, iv). A low titre of antibodies to 3 or 4 other antigens were produced by all rabbits. These largely corresponded to the additional antigens detected by rabbits with the disseminated infection (Fig. 6.8).

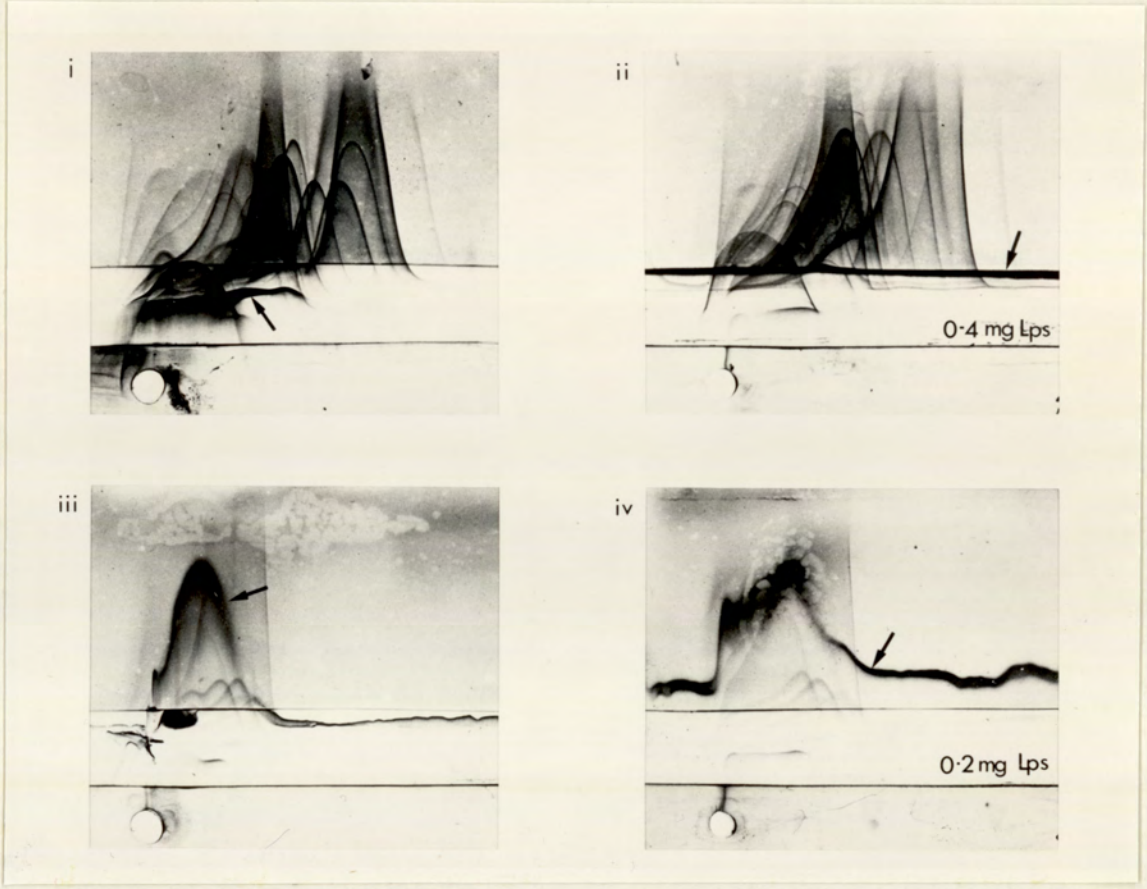


Fig. 6.9. Identification of peak corresponding to LPS using crossed-line immunoelectrophoresis.

P. aeruginosa PAO-1 antigens run against homologous antisera from a rabbit immunized with formalin-killed whole cells (6.9, i and ii) and serum from a rabbit with disseminated peritonitis (6.9, iii and iv).

Intermediate gel contained saline (6.9, i and iii) or 0.4mg and 0.2mg LPS (6.9, ii and iv respectively). LPS was extracted from *P. aeruginosa* PAO-1.

Note the precipitin line of identity in 5.9,ii and iv (marked with arrow).

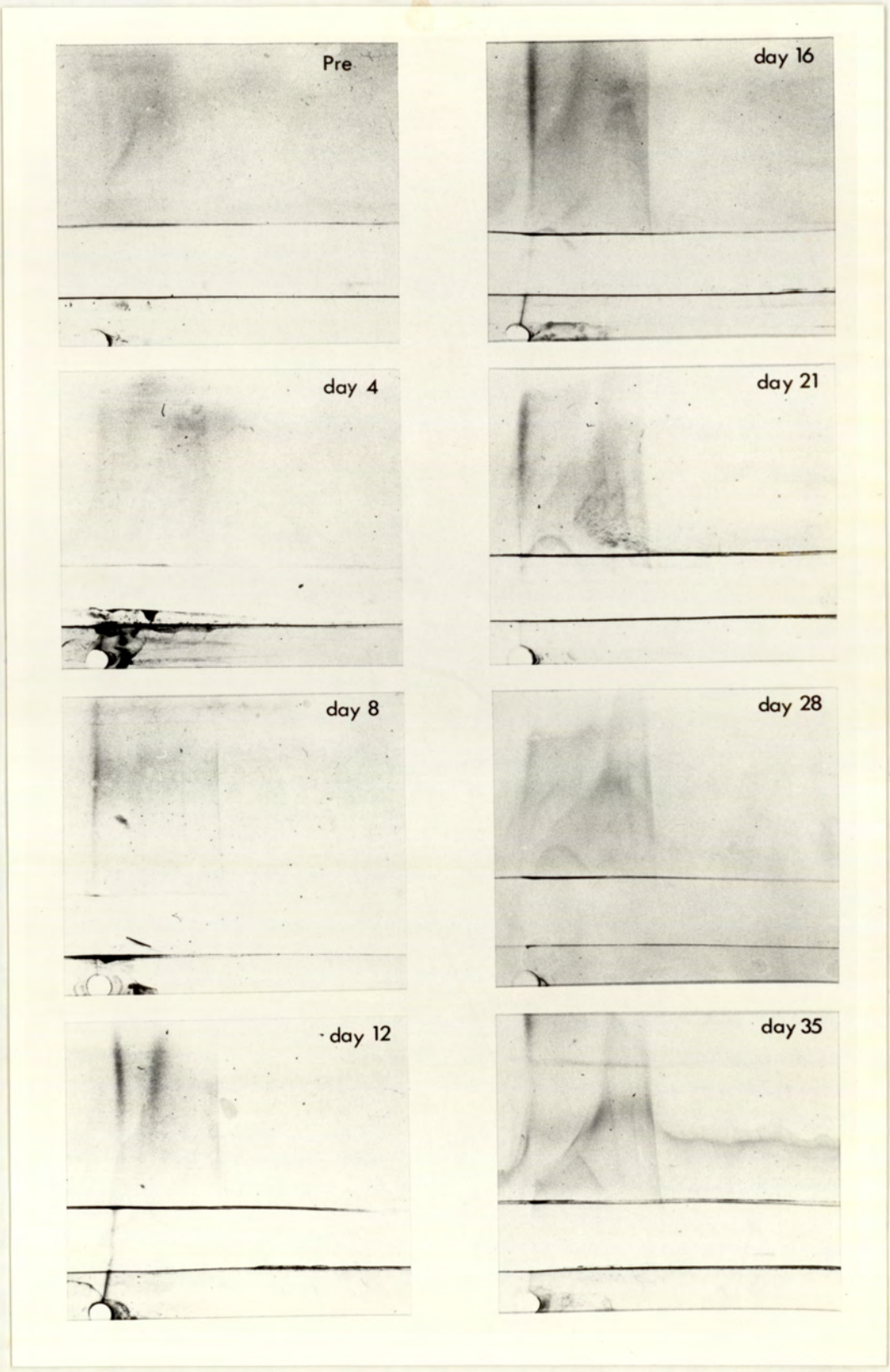


Fig. 6.10, i, ii, iii and iv. Crossed immunoelectrophoresis of *P. aeruginosa* PAO-1 antigens (120µg protein) run against sequential serum samples from 4 rabbits (6.10, i, ii, iii and iv respectively) with biomaterial-associated infection.

'Pre' refers to pre infection serum.

'Days' refer to number of days after onset of infection.

Fig. 6.10, ii.

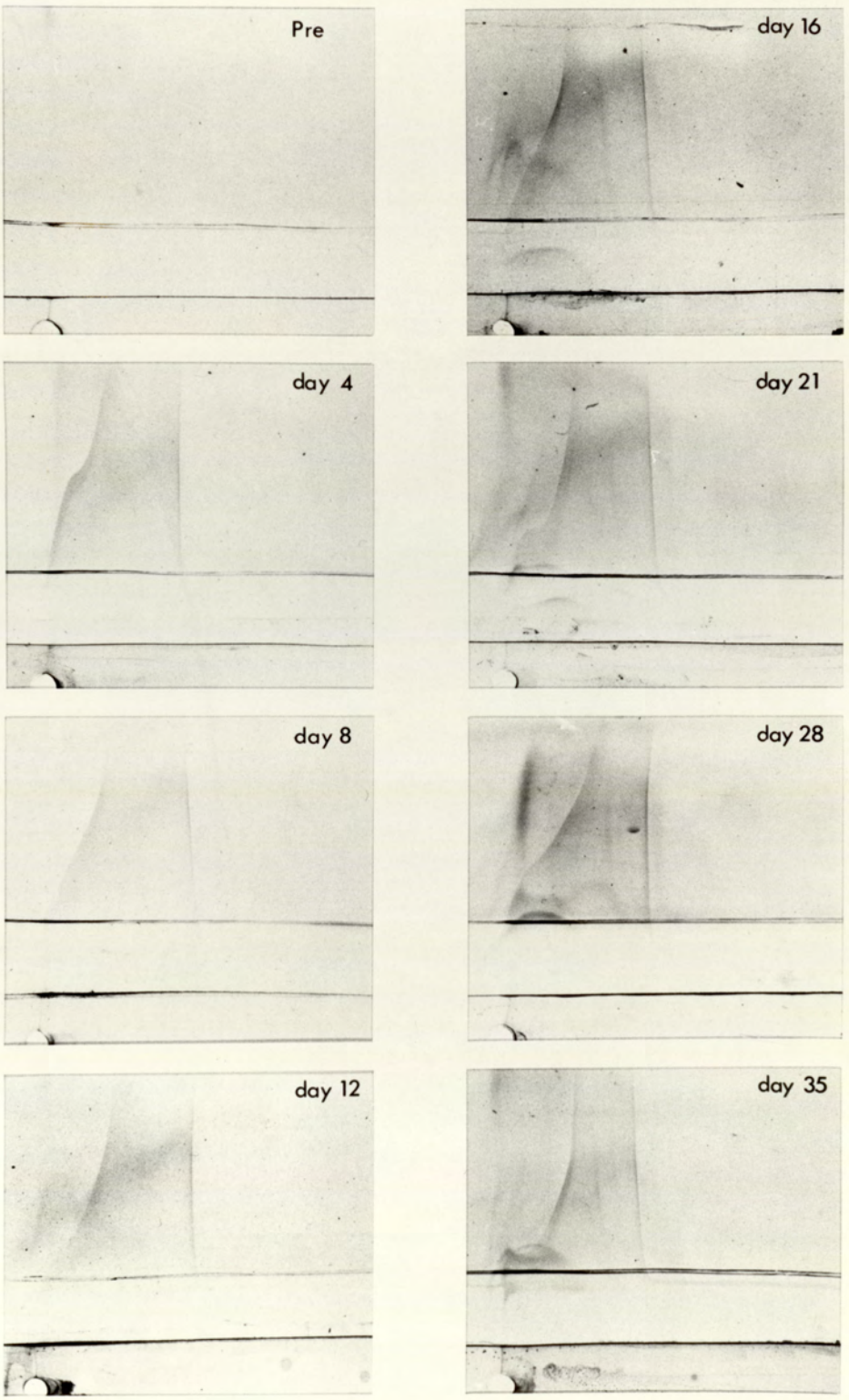


Fig. 6.10, iii.

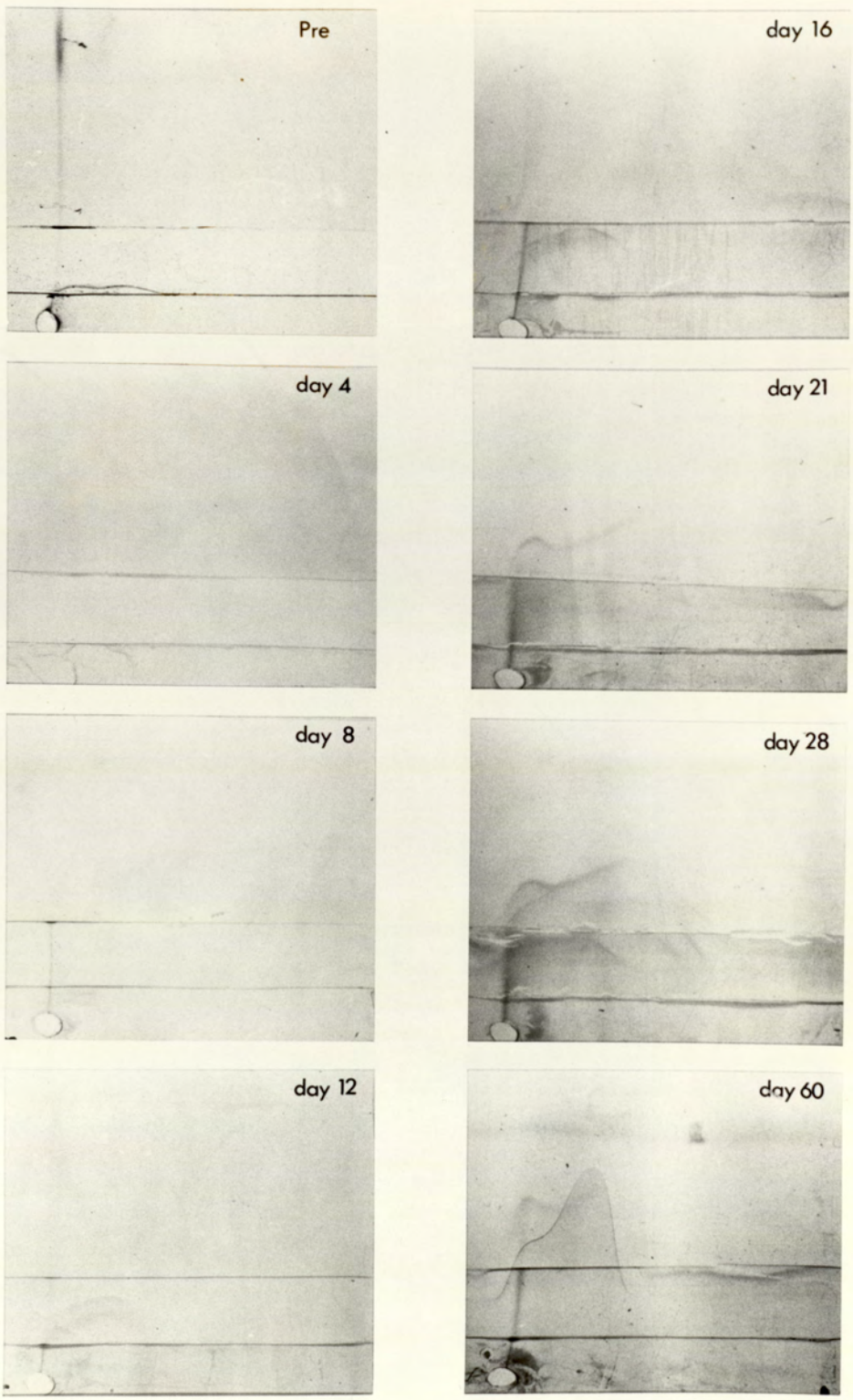
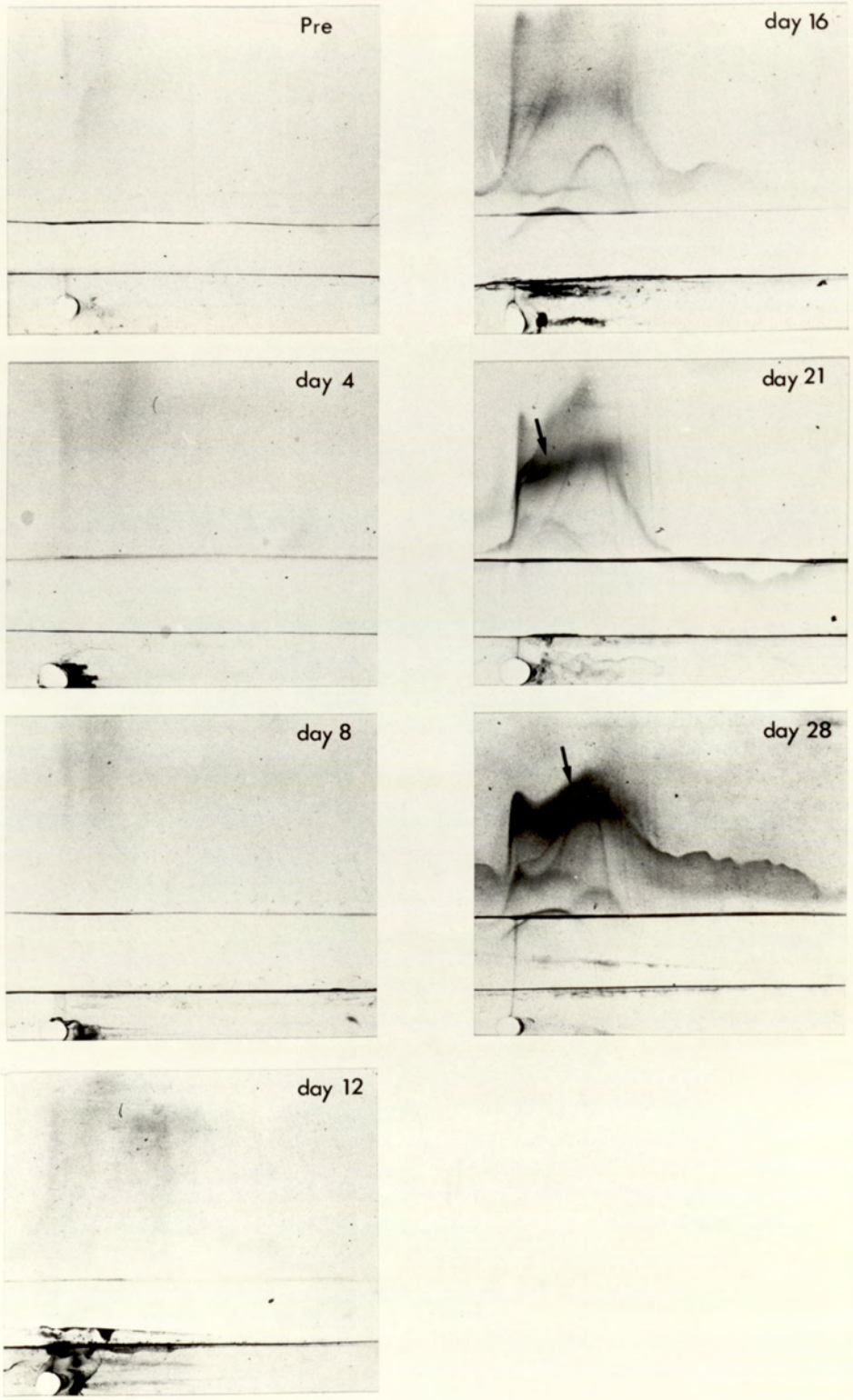


Fig. 6.10, iv.

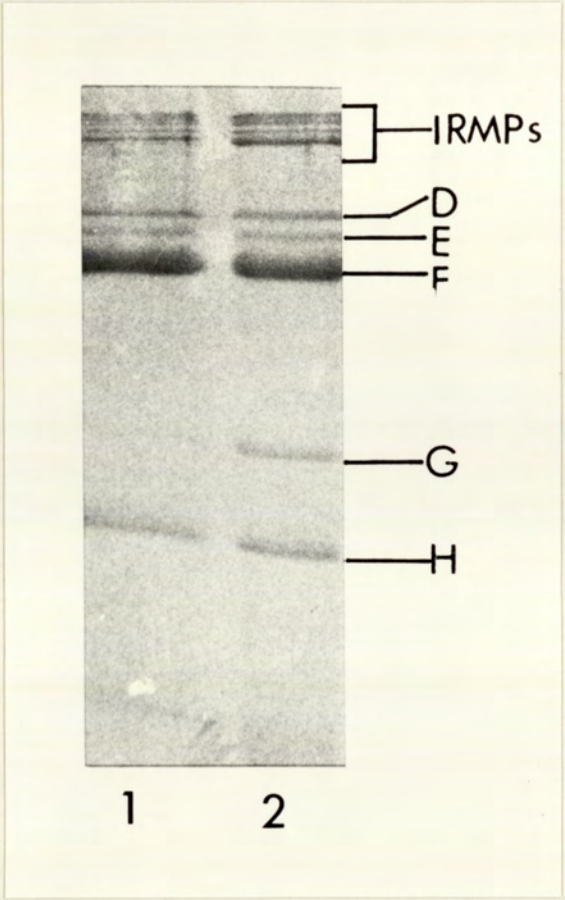


6.3. SDS-PAGE of OMPs and LPS from surface grown and planktonic cells of *P. aeruginosa*.

Preliminary experiments were performed to determine if OMP and LPS profiles of cells cultivated in a liquid medium differed from those of cells grown on the surface of a biomaterial. Flasks containing CDM-Fe and sterile Silastic sheeting were inoculated with *P. aeruginosa* PAO-1 from an overnight culture and cultivated for 24 h. The Silastic sheeting was removed, washed 3 times in sterile PBS to remove loosely attached cells and the remaining sessile population scraped from the surface. OMs were prepared from these cells and from the planktonic cells in the liquid medium, as described in section 3.2.1. The 2 OM preparations were analyzed by SDS-PAGE and stained with Coomassie brilliant blue (Fig. 6.11, i). The familiar OMP profiles were evident in both cases with additional, high molecular weight iron-regulated OMPs (IRMPs) present in the region 77-101K. The only minor difference between the 2 preparations was the apparent reduced expression of protein G in the OM of the sessile bacteria (lane 1) compared to the planktonic cells. Little is understood about the function of protein G although there is some evidence that it is expressed to a lesser degree under iron-limited conditions (see section 4.2; personal communication J. Smith) and under Mg^{2+}- and NH_4^+-limited conditions (Nicas and Hancock, 1980). The significance of this observation is not known.

When the two OM preparations were treated with proteinase-K, separated by SDS-PAGE and silver-stained, a characteristic LPS ladder pattern was observed for both samples (Fig. 6.11, ii), again demonstrating a high degree of heterogeneity amongst LPS molecules. The profiles obtained resembled those reported by Lam *et al* (1987) who stained separated *P. aeruginosa* PAO-1 LPS with silver and Kuzio and Kropinski (1983), who used phosphotungstic - Coomassie blue as a stain. More than 50 species

6.11, i.



6.11, ii.

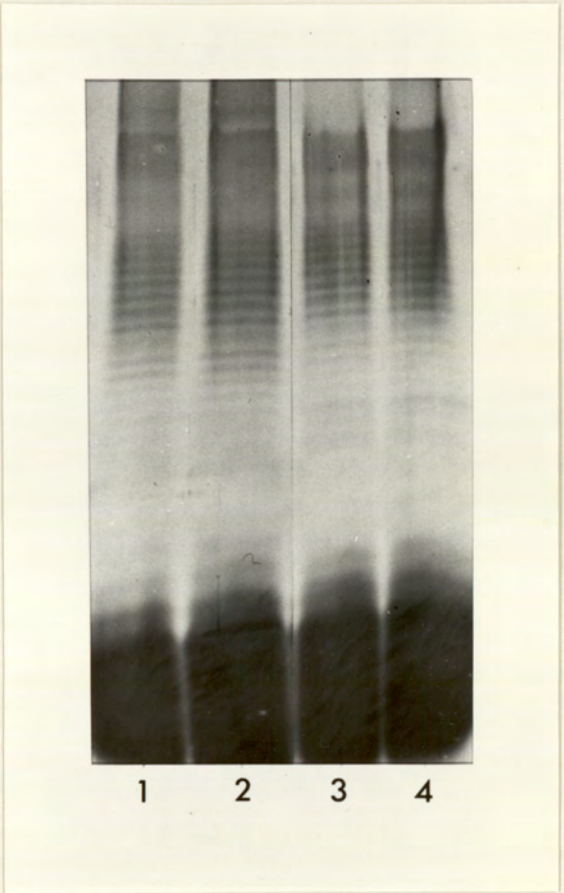


Fig. 6.11, i. OMP profiles of *P. aeruginosa* PAO-1 grown on the surface of Silastic sheeting (sessile; lane 1) or in liquid CDM-Fe (planktonic; lane 2). Proteins stained with Coomassie blue. Molecular weights in kilodaltons.

Fig. 6.11, ii. Proteinase-K digests of OMs from *P. aeruginosa* PAO-1 grown on the surface of Silastic sheeting (sessile; lanes 1 and 2) or in liquid CDM-Fe (planktonic; lanes 3 and 4). LPS profiles stained with silver.

of LPS were observed, and like LPS from *Escherichia coli*, the profile was characterized by enhancement of specific regions (Goldman and Leive, 1980). On comparison, there were no major differences between the profiles obtained from sessile or planktonic cells, although it appeared that there may be a slightly higher proportion of molecules with intermediate length 'O' side chains in the surface-grown bacteria (lanes 1 and 2) compared to liquid-grown cells (lanes 3 and 4).

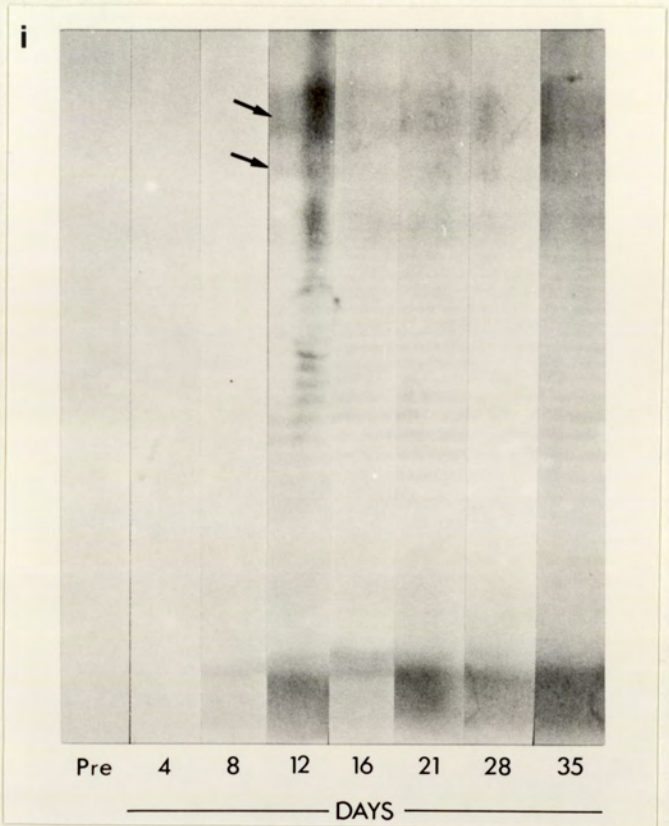
It is clear that the cells in these sessile populations do not reflect very closely those which grew *in vivo* on the implanted devices. The former were not exposed to host defence mechanisms, or bathed in host fluids. Neither were they growing for the same length of time as the *in vivo* cells and growth rates may have varied. The study does indicate, however, that growth on the surface of a biomaterial per se has little overall effect on the profiles of the OM antigens studied by SDS-PAGE.

6.4. Sequential rabbit humoral immune response to *P. aeruginosa* antigens studied by immunoblotting.

6.4.1. Response to LPS.

The sensitive technique of immunoblotting was used to further study the immune response of rabbits in the 2 models of infection, so enabling recognition of the separated components of LPS to be investigated. Proteinase-K digests of *P. aeruginosa* PAO-1 OMs prepared from cells grown in CDM-Fe were separated by SDS-PAGE, transferred to NC paper and probed with the rabbit serum. The IgG response was visualized with protein-A peroxidase. The characteristic results for 2 rabbits with disseminated peritonitis are shown in Fig. 6.12, i and ii. No reaction to LPS was observed with pre infection serum (labelled 'Pre'), but in both rabbits low molecular weight material or lipid A-core oligosaccharide, migrating to the bottom of the gel, was detected 8 days after onset of infection. In one rabbit (Fig. 6.12, i) this was followed only 4 days later by a response to all the species of LPS molecules. This included very high molecular weight smooth LPS material which had not resolved into separate bands and was seen as darkened regions of the immunoblot (marked with arrows). The 2nd rabbit (Fig. 6.12, ii) detected high molecular weight material in addition to the core/lipid A, 12 days after onset of infection. A response to intermediate molecular weight species of LPS was seen developing more strongly by day 21. The detection of blotted LPS by a rabbit immunized with formalin-killed whole cells of *P. aeruginosa* PAO-1 is shown in the last lane (Fig. 6.12, ii, labelled 'Imm') demonstrating a very strong reaction to all species of the separated LPS such that many of the bands were obscured.

When similar studies were carried out with serum from rabbits with biomaterial-associated infection the LPS antigen was barely detected. Typical results are shown in Fig. 6.13 which shows only a weak response to the core-lipid A band at the bottom of the gel and not until day 28.



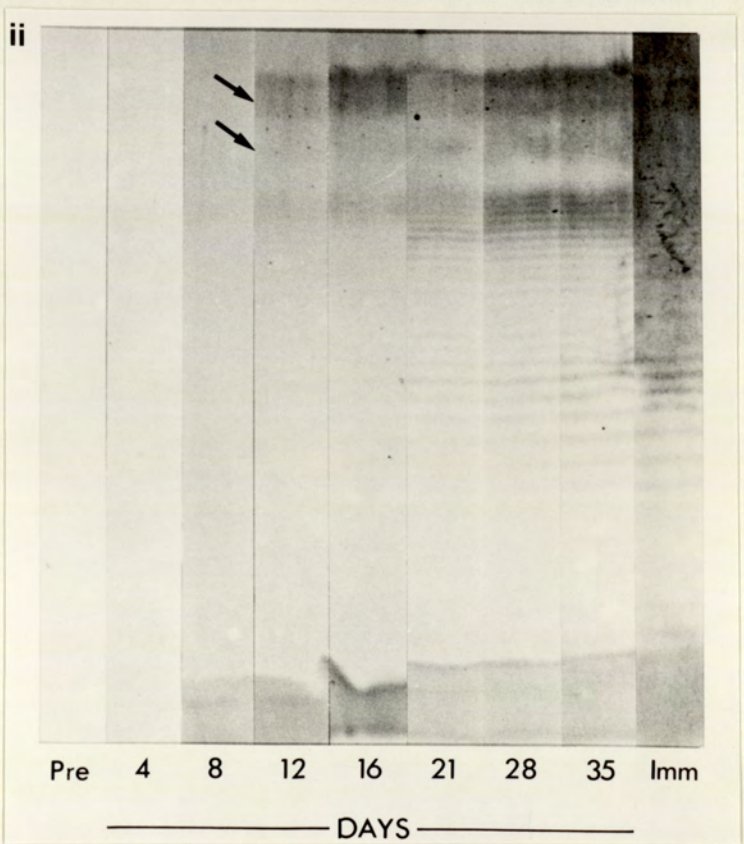


Fig. 6.12. i and ii. Strip immunoblots of *P. aeruginosa* PAO-1 LPS (proteinase-K digested OMs) probed with sera taken sequentially from 2 rabbits (i and ii respectively) with disseminated peritonitis. Sera diluted 1 in 20 in TBS/Tween. IgG response detected with protein-A peroxidase.
'Pre' refers to pre infection serum.
'Days' refer to number of days after onset of infection.
'Imm' refers to homologous rabbit antisera raised against formalin-killed whole cells of *P. aeruginosa* PAO-1.

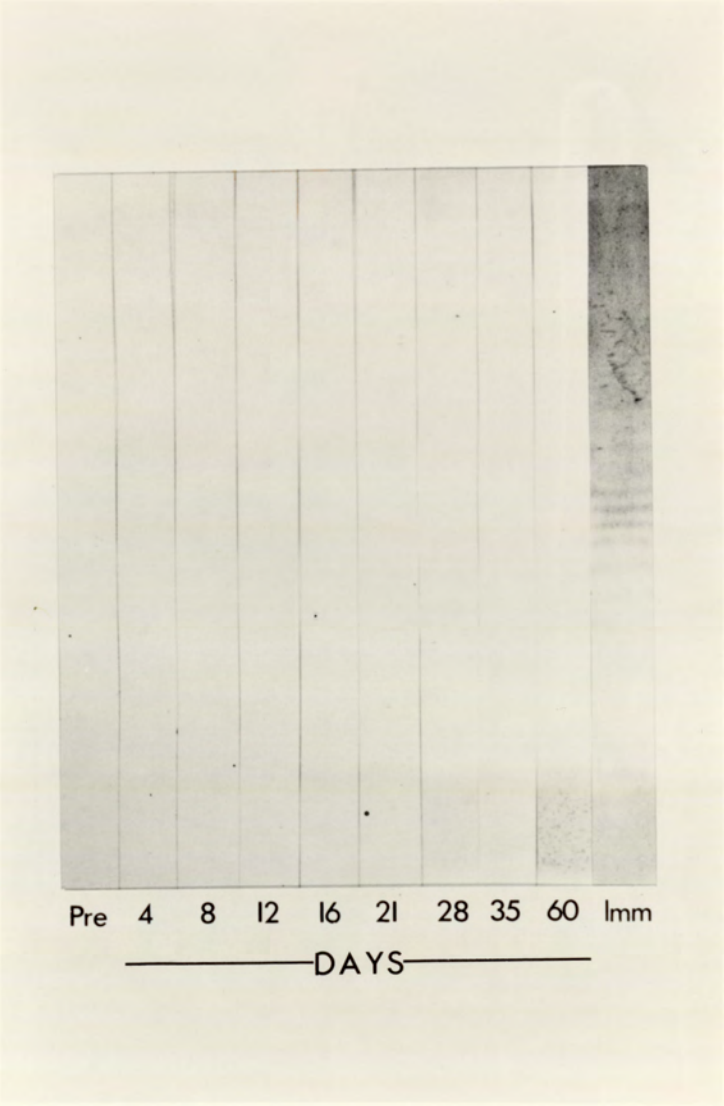


Fig. 6.13. Strip immunoblots of *P. aeruginosa* PAO-1 LPS probed with sera taken sequentially from a rabbit with biomaterial- associated infection. Sera diluted 1 in 20 in TBS/Tween. IgG response detected with protein-A peroxidase.

'Pre' refers to pre-infection serum.

'Days' refer to number of days after onset of infection.

'Imm' refers to homologous rabbit antisera raised against formalin-killed whole cells of *P. aeruginosa* PAO-1.

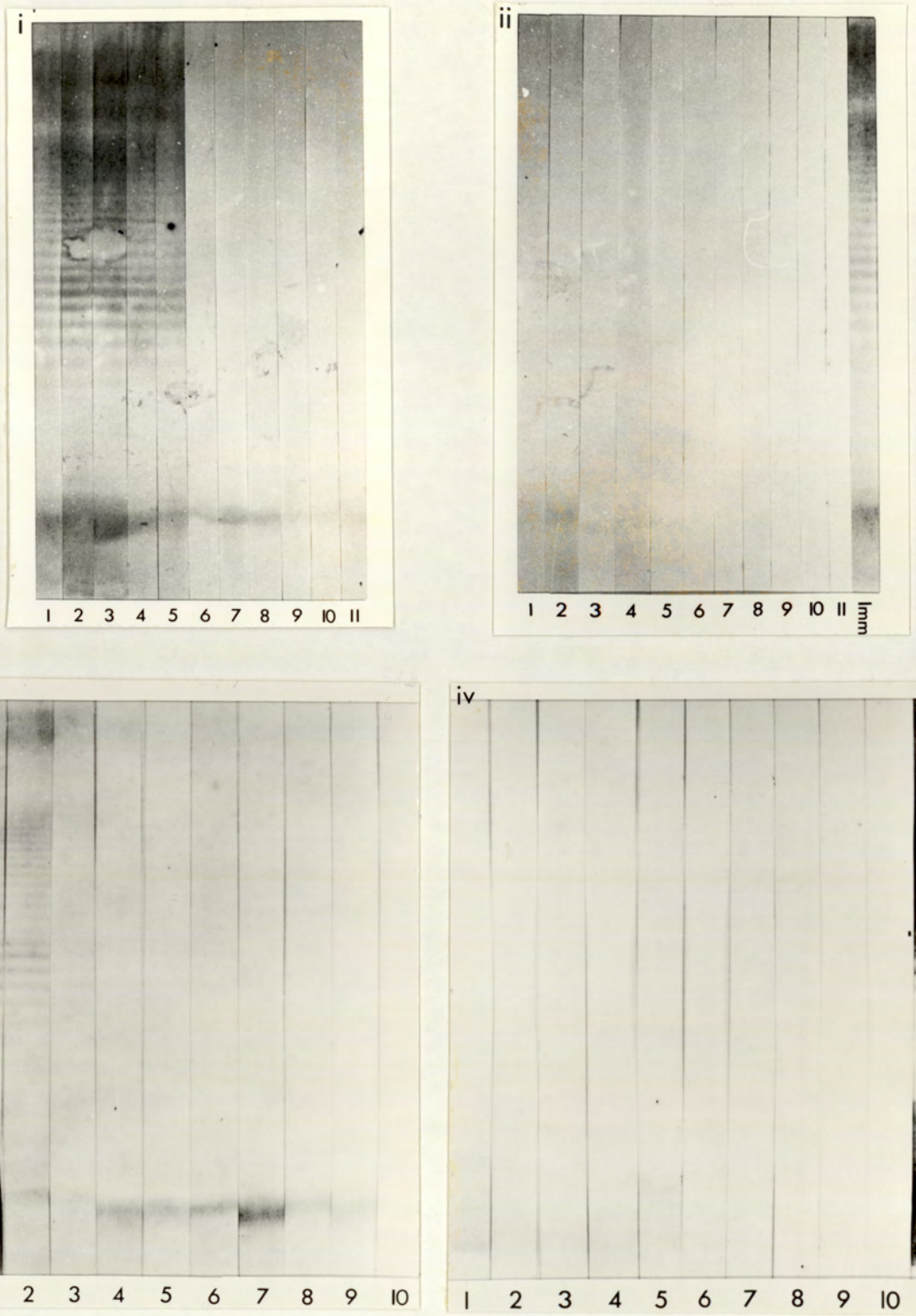


Fig. 6.14. Strip immunoblots of *P. aeruginosa* PAO-1 LPS probed with 35-day (i and ii) and 60-day (iii and iv) sera from a rabbit with disseminated peritonitis (i and iii) or with biomaterial associated infection (ii and iv). Sera diluted in TBS/Tween as follows:

Lane	Dilution	Lane	Dilution
1	1:10	6	1:200
2	1:20	7	1:400
3	1:40	8	1:800
4	1:60	9	1:1000
5	1:100	10	1:2000
		11	1:5000

IgG response detected with protein-A peroxidase.
'Imm' refers to a rabbit immunized with formalin killed PAO-1 whole cells.

Even at day 60, 4 weeks after numbers approaching 10^9 cells were present on the disc surfaces, the response to LPS was low.

All of the immunoblots described thus far were probed with serum at a dilution of 1 in 20. In an attempt to quantify the antibody titre to LPS using this technique, sequential dilutions of sera obtained 35 (6.14, i and ii) and 60 (6.14, iii and iv) days after onset of infection from a representative of the rabbits in both models were used to probe replicate strips of blotted LPS. It is clear that day 35 serum from rabbits with the disseminated infection (Fig. 6.14, i) had a much higher titre of antibodies to LPS than that from rabbits with the biomaterial associated infection (Fig. 6.14, ii), rough LPS still being detected by the former serum at a dilution of 1 in 5000 compared to 1 in 20 for the latter. By day 60, the titre for rabbits with disseminated peritonitis had fallen slightly (Fig. 6.14, iii) and that for rabbits with the biomaterial-associated infection remained low (Fig. 6.14, iv). These observations further substantiate the crossed immunoelectrophoresis results described in section 6.2.

6.4.2. Response to OMPs.

Immunoblotting was additionally used to investigate the detection of OMPs by serum antibodies of rabbits in both models of infection. OMs of *P. aeruginosa* PAO-1 cultivated in CDM-Fe were separated by SDS-PAGE, transferred to NC paper and replicate strips probed with rabbit serum. This was followed by protein A peroxidase to detect the IgG response. The results obtained with 2 rabbits in the disseminated peritonitis model are shown in Figs. 6.15, i and ii. In all cases the last lane represents an amido black stain of an identical NC strip to confirm qualitative transfer of the OMPs and to locate the position of the bands. Pre-infection serum detected no antigens. The first rabbit (Fig. 6.15, i) showed a weak response to the IRMPs and proteins D and E, 4 days after onset of infection, which

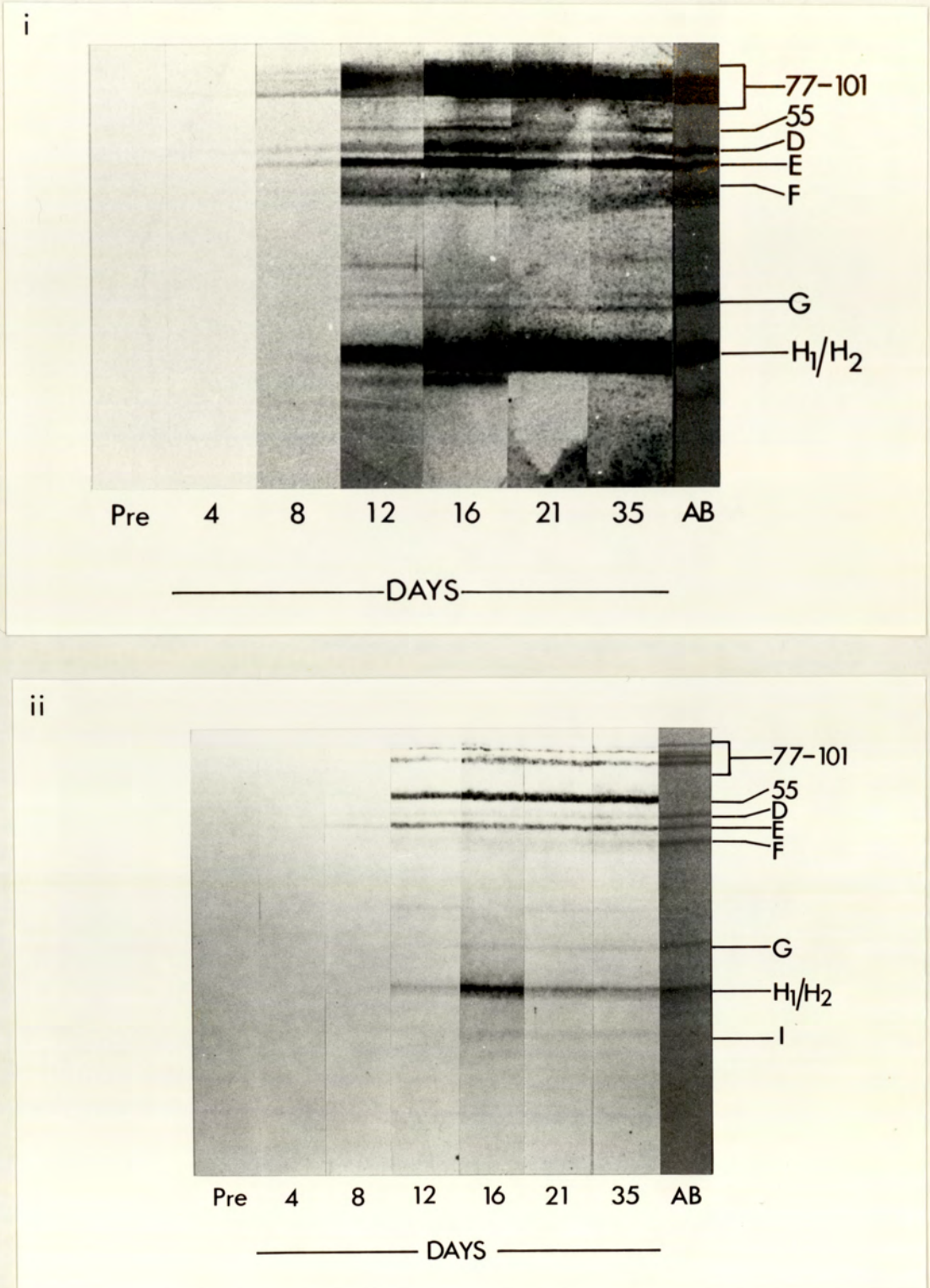


Fig. 6.15, i and ii. Strip immunoblots of *P. aeruginosa* PAO-1 OMPs probed with sera taken sequentially from 2 rabbits (i and ii) with disseminated peritonitis. Sera diluted 1 in 20 in TBS/Tween. IgG response detected with protein-A peroxidase.

'Pre' refers to pre infection serum.

'Days' refer to number of days after onset of infection.

'AB' refers to a replicate NC strip stained with amido black 1% w/v.

had intensified by day 8. All the major OMPs had elicited antibody formation by day 12 and, in addition, an antigen of approximate molecular weight 55K was observed above protein D. This antigen was not detectable on the amido black stain, nor in the Coomassie blue-stained gel (Fig. 6.11) and, therefore, was present in low amounts, but it clearly stimulated antibody production throughout the course of the infection. A particularly marked reaction to proteins H_1/H_2 (predominantly H_1) was evident but not until 12 days after onset of infection. A similar pattern of response was observed with the 2nd rabbit (Fig. 6.15, ii). IRMPs and proteins D and E were weakly detected by day 8 and a response to all the proteins was visible by day 12. The 55K antigen was also strongly recognized along with proteins H1/H2.

The molecular weight of the additional antigen (55K) detected so strongly by both rabbit sera and also seen in one of the burn patients, suggested that it may be flagella protein, present in fairly low levels in the OM preparation after Sarkosyl extraction. In an attempt to verify this, flagella antigen was purified from *P. aeruginosa* PAO-1 by the method of Montie *et al* (1982a) and its purity determined by SDS-PAGE (Fig. 6.16). The purified antigen ran as a single band of approximate molecular weight 55K. Immunoblotting of the flagella protein gave the results shown in Fig. 6.17. Fig. 6.17, i, represents an amido black stain of the transferred proteins and Fig. 6.17, ii, is an identical immunoblot incubated in serum from a rabbit with disseminated infection. In both cases lane 1 refers to *P. aeruginosa* PAO-1 OMs, lane 2, the purified flagella protein alone and lane 3, OMs and flagella protein combined. The purified flagella was detected strongly by serum antibodies (Fig. 6.17, ii, lanes 2 and 3) and appeared to have the same migration pattern as the additional band present in lane 1. Such evidence implies that this band may well represent flagella protein.

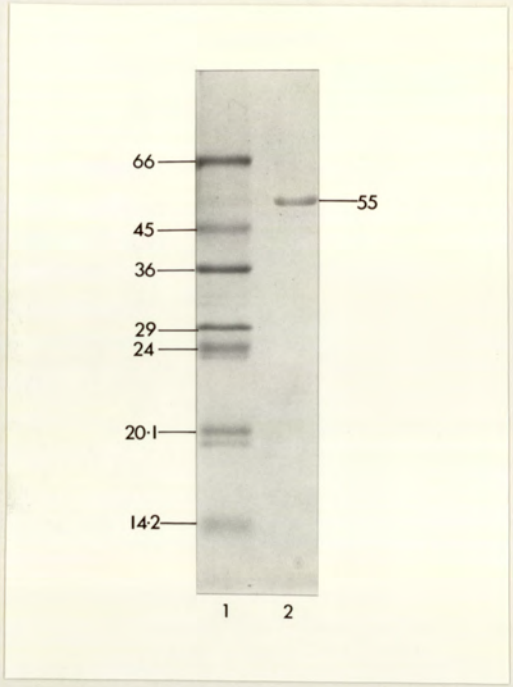


Fig. 6.16. SDS-PAGE of purified flagella protein from *P. aeruginosa* PAO-1 (lane 2) and molecular weight standards as follows: 66K, bovine albumin; 45K, egg albumin; 36K, glyceraldehyde-3 phosphate dehydrogenase; 29K, carbonic anhydrase; 24K, trypsinogen; 20.1K, trypsin inhibitor; 14.2K, α-lactalbumin.

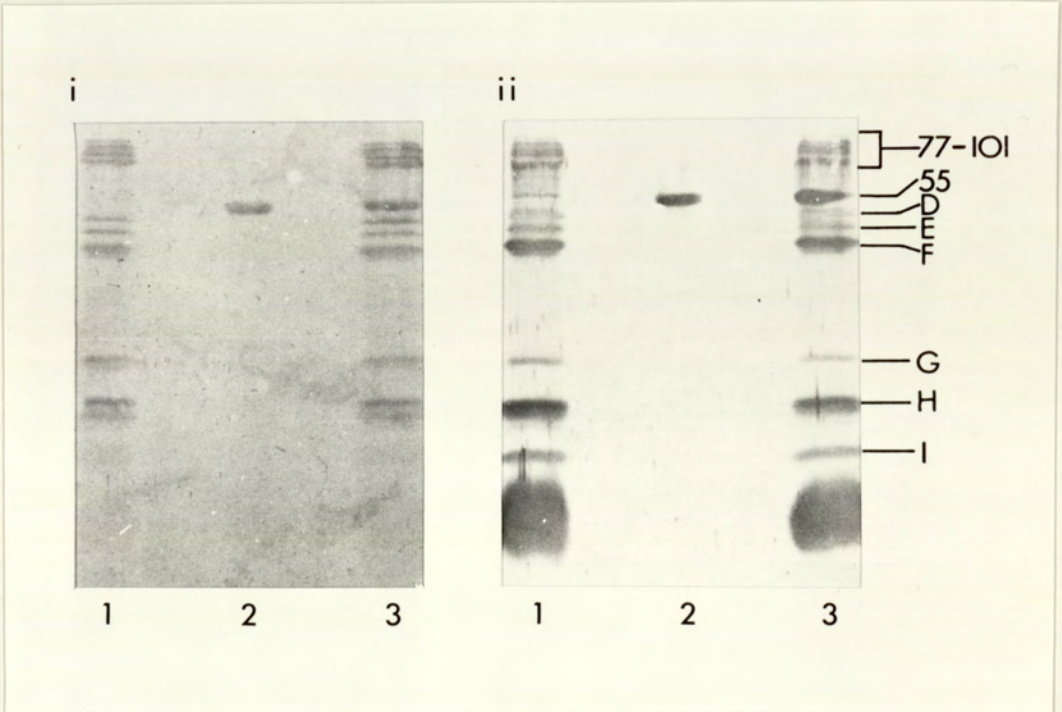


Fig. 6.17, i. *P. aeruginosa* PAO-1 OMPs (lane 1), purified flagella protein (lane 2) and OMs + flagella protein (lane 3) separated by SDS-PAGE, transferred to NC paper and stained with amido black 1% w/v.

Fig. 6.17, ii. Immunoblot of the proteins described in Fig. 6.17, i, probed with serum from a rabbit with disseminated peritonitis, 28 days after onset of infection. IgG response detected with protein-A peroxidase.

In order to determine the contribution of contaminating LPS on the immunoblots to the banding pattern observed in Figs. 6.15, i and ii, anti-LPS antibodies were removed from the serum by immunoprecipitation as described in section 2.7.4. Fig. 6.18 demonstrates the results of probing replicate LPS immunoblots with serum before (lane 1) and after (lane 2) absorption with LPS. The serum was taken on day 35 from a rabbit with disseminated peritonitis, and was previously shown to have a high titre of anti-LPS antibodies (Fig. 6.12, ii). The LPS ladder was clearly visible with the untreated serum (lane 1) but absent after absorption (lane 2) indicating, as in section 5.2.1, that the procedure had effectively reduced the titre of anti-LPS antibodies to a level which no longer significantly detected LPS on the immunoblot. Immunoblotting of the OMPs shown in Fig. 6.15 was then repeated with the LPS-absorbed sera from the same 2 rabbits (Figs. 6.19, i and ii). The pattern of recognition was essentially the same as that seen with the untreated sera. It appeared that the intensity of the response, especially in the upper part of the blot may have been reduced to a degree indicating that high molecular weight LPS present in that region of the blot may have somewhat enhanced the reaction to the protein bands in sera containing anti-LPS antibodies. Following removal of these antibodies the IRMPs were still detected and by only 4 to 8 days after onset of infection.

LPS-absorbed sera from 2 rabbits with biomaterial-associated infection were then used to probe OMP immunoblots (Fig. 6.20, i and ii). With one rabbit (Fig. 6.20, i), although immunoglobulins against IRMPs and proteins D and E were present in serum 8 days after onset of infection, a full response to the other OMPs was not seen until day 35 when protein F and H were more strongly recognized. The flagella again stimulated a strong immune response, 16 days post onset. The second rabbit, detected IRMPs at an early stage, but compared to rabbits with the disseminated peritonitis

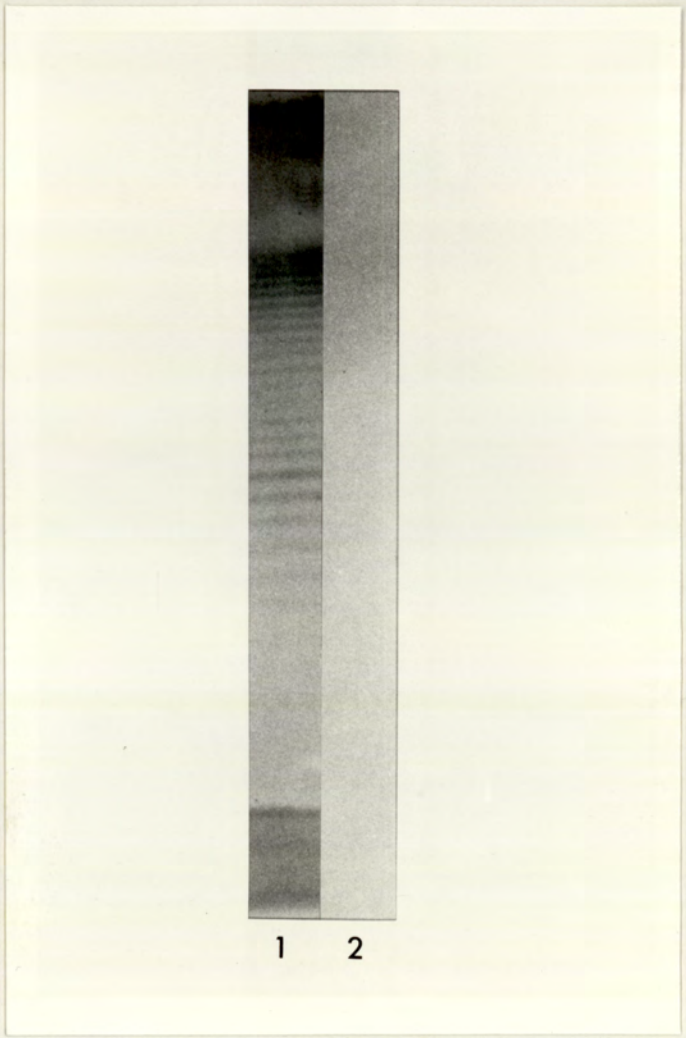


Fig. 6.18. Strip immunoblots of *P. aeruginosa* PAO-1 LPS (proteinase-K digested OMs) probed with untreated serum (lane 1) and LPS-absorbed serum (lane 2) from a rabbit with disseminated peritonitis (day 35). IgG response detected with protein-A peroxidase.

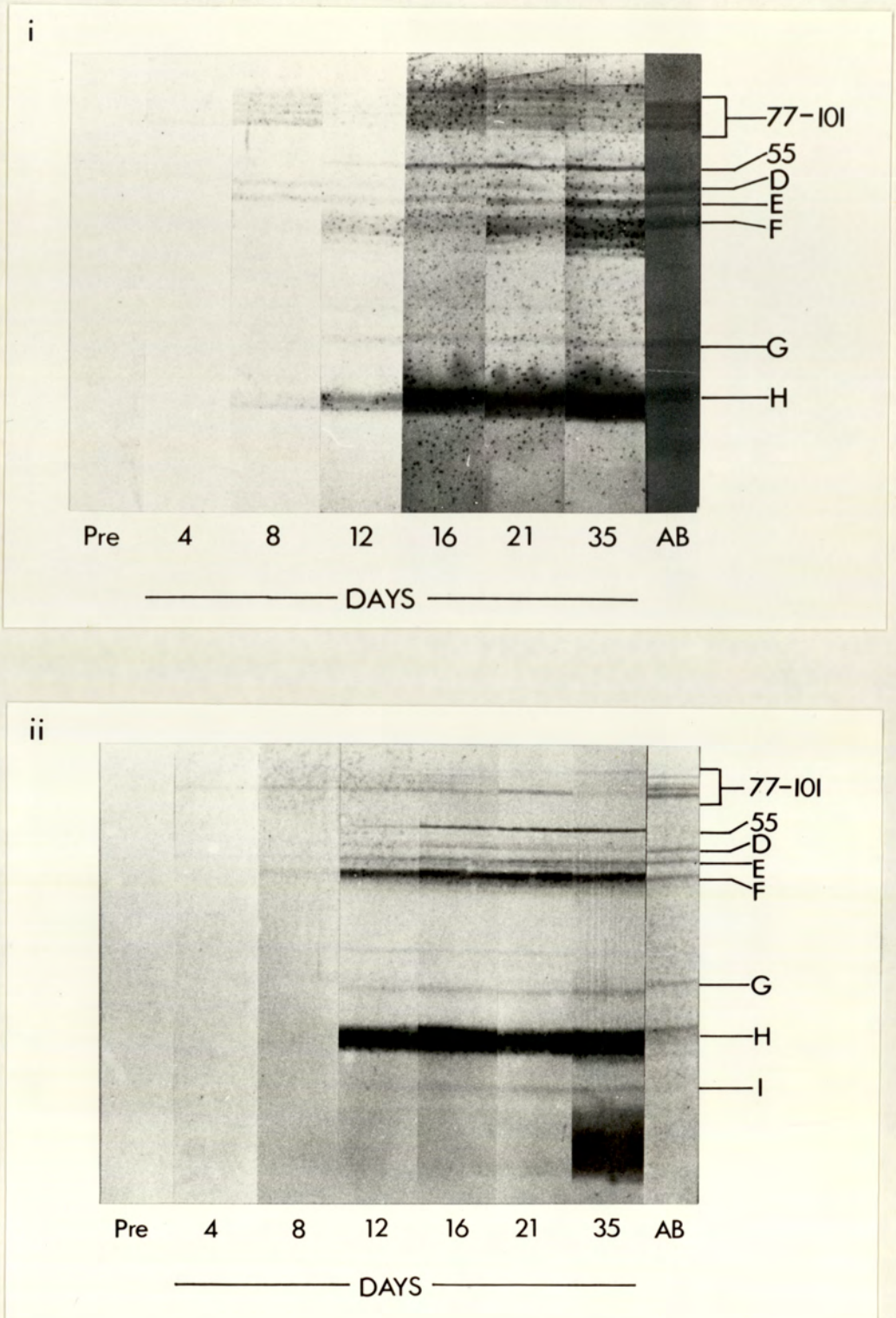


Fig. 6.19, i and ii. Strip immunoblots of *P. aeruginosa* PAO-1 OMPs probed with LPS-absorbed sera taken sequentially from 2 rabbits (i and ii) as described in Fig. 6.15 with disseminated peritonitis. Sera diluted 1 in 20 in TBS/Tween. IgG response detected with protein-A peroxidase.

'Pre' refers to pre infection serum.

'Days' refer to number of days after onset of infection.

'AB' refers to a replicate NC strip stained with amido black 1 % w/v.

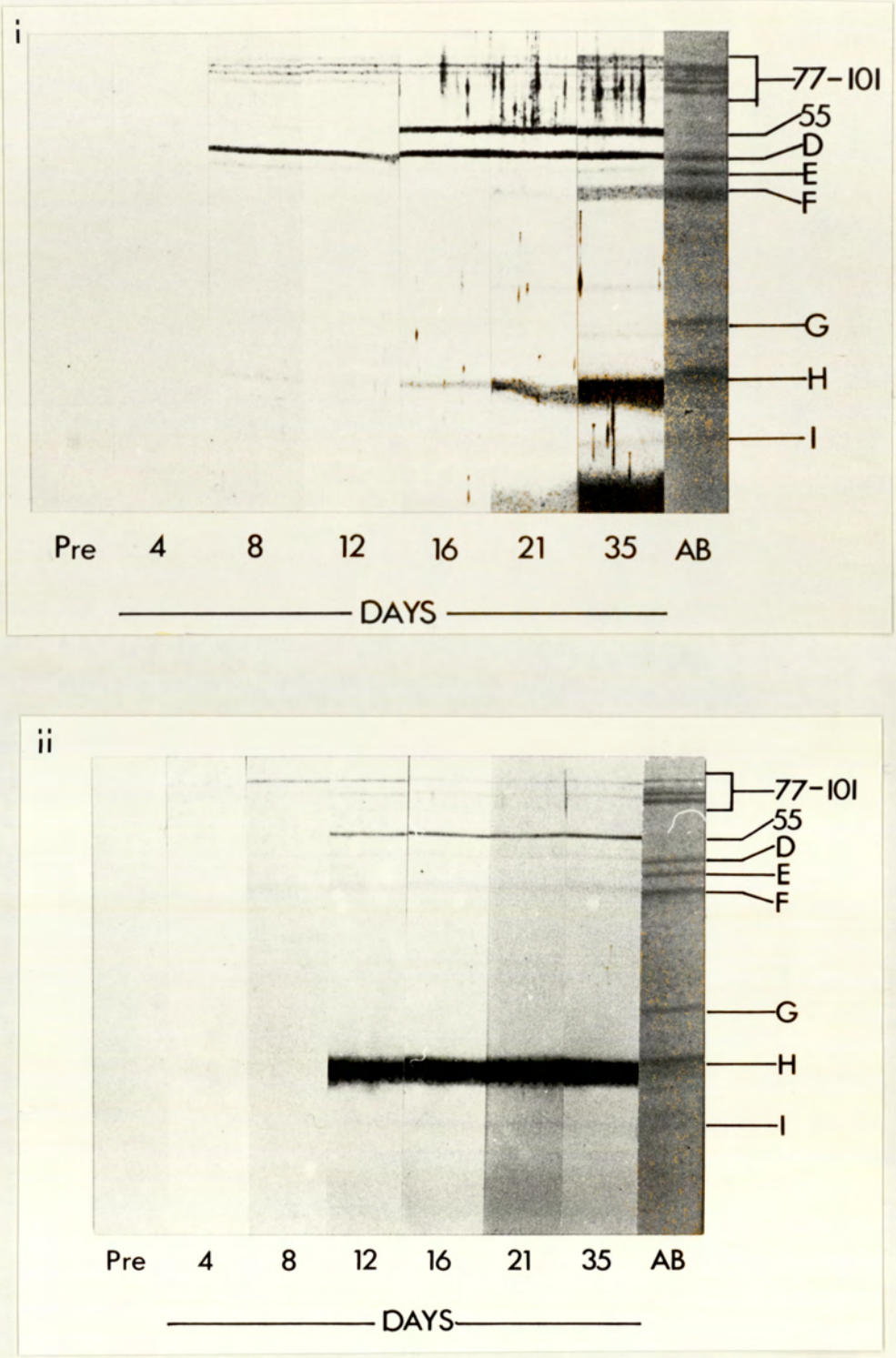


Fig. 6.20, i and ii. Strip immunoblots of *P. aeruginosa* PAO-1 OMPs probed with LPS-absorbed sera taken sequentially from 2 rabbits (i and ii) with biomaterial associated infection. Sera diluted 1 in 20 in TBS/Tween. IgG response detected with protein-A peroxidase.

'Pre' refers to pre infection serum.

'Days' refer to number of days after onset of infection.

'AB' refers to a replicate NC strip stained with amido black 1% w/v.

(Figs. 6.19, i and ii), other OM antigens were less stongly detected. The exception was protein H to which a marked reaction was noted.

Again, in an attempt to use immunoblotting to yield more quantitative results, OMP blots were probed with sequential dilutions of LPS-absorbed serum obtained 35 days after onset of infection from a representative of the rabbits in both models of infection to compare the degree of response. From Fig. 6.21 it can be seen that following the disseminated infection day 35 serum detected the majority of OMPs up to a dilution of 1 in 200, and that flagella, protein H and one of the IRMPs were still recognized at a dilution of 1 in 1000. Day 35 serum following biomaterial-associated infection only detected the total array of OMP antigens at a dilution of 1 in 10, with flagella antigen alone seen at 1 in 200. Similar results were obtained with day 60 serum from both rabbits (data not shown).

A more accurate determination of serum antibody titres to *P. aeruginosa* PAO-1 may have been obtained using an enzyme-linked immunosorbent assay (ELISA). However, to assess the response to the individual proteins they would each have had to be purified. As such, the immunoblotting method, albeit less accurate, was sufficient to demonstrate that a greater immune response had been mounted to the OMPs by rabbits with disseminated peritonitis than those with biomaterial-associated infection. Furthermore, flagella protein was seen to be highly immunogenic, antibodies to the antigen being detected at dilutions far greater than other OMPs despite the low level of the antigen present on the blot, (too low to be stained with amido black).

The presence of antibodies locally at the site of infection was investigated by recovering samples of pus or peritoneal fluid from the rabbits with disseminated peritonitis. The latter were concentrated by lyophilization, resuspended in TBS/Tween (section 3.5.1) and used in place of serum to probe immunoblots. It was not possible to immunoprecipitate

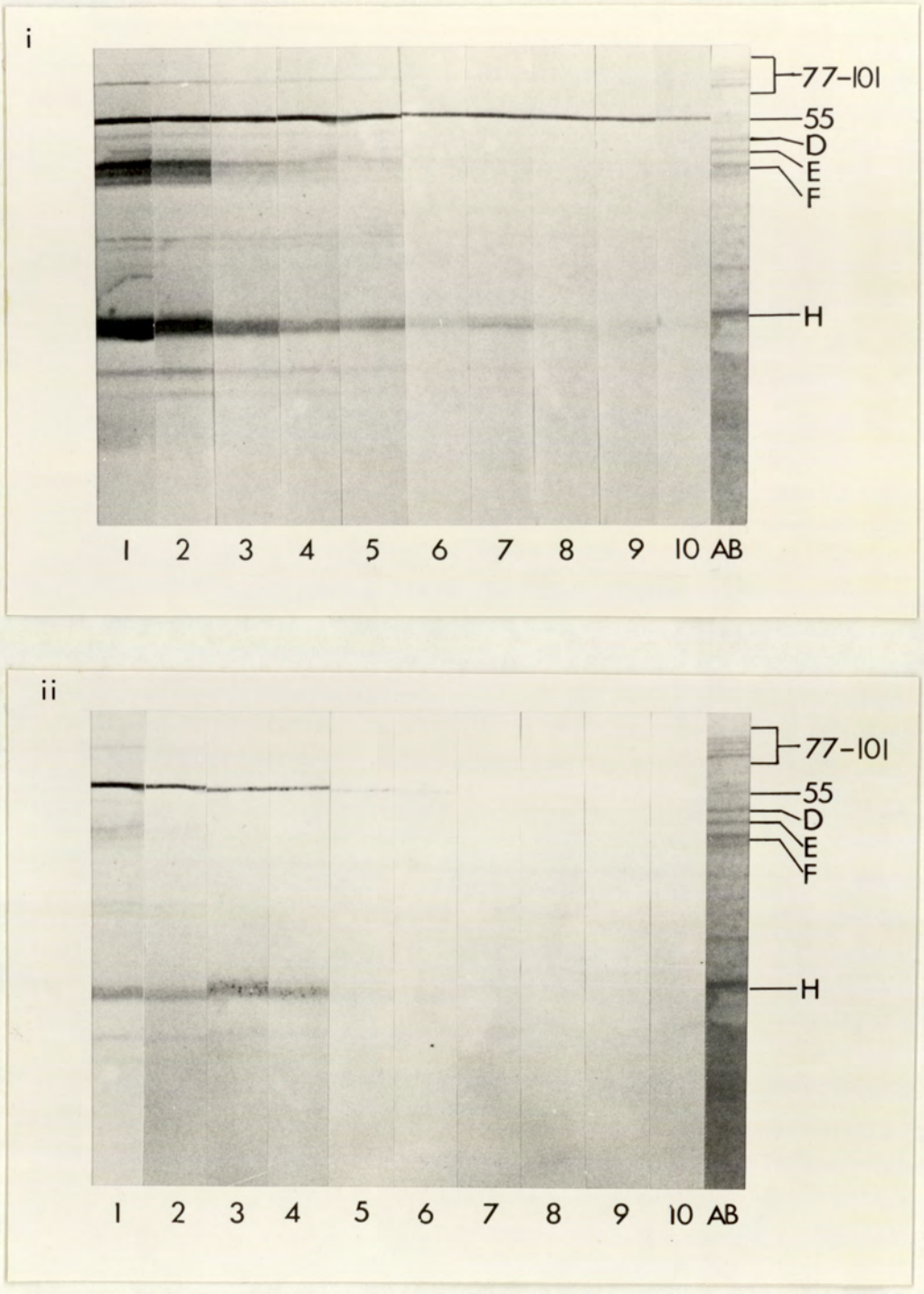


Fig. 6.21, i and ii. Strip immunoblots of *P. aeruginosa* OMPs probed with day 35 serum from a rabbit with disseminated peritonitis (i) or biomaterial associated infection (ii). Serum diluted in TBS/Tween as follows:

Lane	Dilution	Lane	Dilution
1	1:10	6	1:200
2	1:20	7	1:400
3	1:40	8	1:800
4	1:60	9	1:1000
5	1:100	10	1:2000

IgG response detected with protein-A peroxidase.

'AB' represents a replicate NC strip stained with amido black 1% w/v.

anti-LPS antibodies from the pus samples owing to the physical nature of these preparations. Fig. 6.22, lane 1 shows the result of incubating the blot in peritoneal fluid obtained from a rabbit who died 3 days after initiation of disseminated infection. The antibodies present in the fluid were predominantly directed against the IRMPs and proteins D and E. This represents the early immune response to the peritonitis and identifies the antibodies released into the peritoneal fluid at the site of the infection. Lanes 2 and 3 refer to antibodies in pus samples obtained from rabbits 4 and 10 days after onset of infection respectively. Therefore, the pattern of response closely reflected that observed with serum: IRMPs and proteins D and E eliciting early antibody formation and a response to protein F and H developing at a later stage.

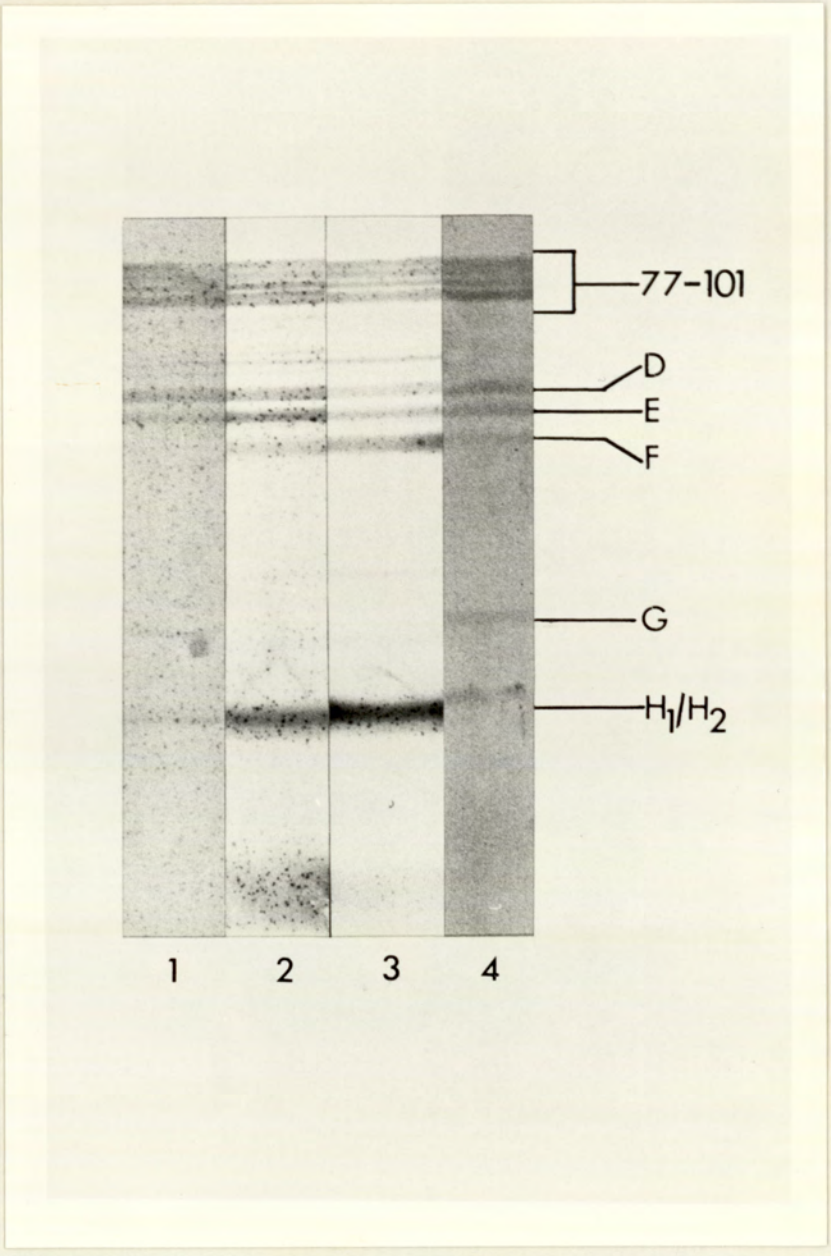


Fig. 6.22. Strip immunoblots of *P. aeruginosa* PAO-1 OMPs probed with peritoneal fluid obtained 3 days after onset of infection (lane 1) and pus samples obtained 4 and 10 days after onset of infection (lanes 2 and 3, respectively). Fluid and pus samples were concentrated by lyophilization.

IgG response detected with protein-A peroxidase.

Lane 4 represents a replicate NC strip stained with amido black 1% w/v.

6.5 Discussion

Two rabbit models of infection were established in this study which differed in several respects. The first, termed the disseminated peritonitis, was characterized by an acute phase of infection in which a widespread peritonitis resulted. This implied that at some early stage, planktonic, free-floating bacteria were released in numbers sufficient to spread to and colonize tissues distal from the initial site of infection. Unfortunately, it was not possible to determine the number of bacteria present in the peritoneum at the height of infection. Certain rabbits in this model died within 24 h of implantation, probably killed by the effects of LPS toxicity. The remaining rabbits successfully cleared the infection in the peritoneal cavity but were unable to eradicate the bacteria adherent on the disc.

The second rabbit model involved a predominantly sessile population of bacteria growing as an adherent microcolony on the surface of the Silastic disc implants. Any planktonic bacteria released were presumably low in numbers and hence effectively destroyed by macrophages and PMNs prolific in the peritoneal cavity (Daems, 1980; Jennings *et al*, 1980) such that there was no evidence of a disseminated peritonitis. A chronic, localized infection resulted. Bacteria multiplied within the adherent biofilm to the order of 10^{8} to 4 x 10^{9} cells by the 4th week and possibly sooner (it would have been beneficial to ascertain bacterial numbers at an earlier stage). The biofilm mode of growth was confirmed by SEM and TEM, which revealed groups of bacteria embedded in the dehydrated remains of the biofilm glycocalyx that had enveloped them.

The exact nature of the glycocalyx material seen to surround surface-growing cells of *P. aeruginosa* has been difficult to define (Brown, 1975; Irvin and Ceri, 1985). It is thought to be partially composed of exopolysaccharides which can be visualized with the polyanionic stain,

ruthenium red (Luft, 1971) and possibly other components including proteins, lipids, LPS constituents (Pier, 1985). These are all of bacterial origin, however, polysaccharides and detritus of host origin may also be present (Costerton *et al*, 1983). Exopolysaccharide production is now considered to be a property of most *P. aeruginosa* isolates depending on environmental conditions, not only those strains termed "mucoid" by virtue of their colonial morphology on BHI agar (Costerton *et al*, 1983; Chan *et al*, 1984). A more detailed account of the exopolysaccharides produced was given in section 1.2.4.

It appears that the formation of adherent biofilms is a mode of growth which *P. aeruginosa* may adopt in several different habitats, both in the medical environment including the surfaces of a variety of prosthetic devices (Marrie and Costerton, 1983a; Gristina and Costerton, 1985; Nickel *et al*, 1985), and in its natural aquatic habitats on submerged surfaces (Geesey *et al*, 1978). In the case of the former, these adherent cells are extremely persistent, such infections rarely resolving unless the colonized biomaterial is removed (Coventry, 1975; Gristina and Costerton, 1985). Indeed this factor was demonstrated by the rabbit model used in this study.

Crossed immunoelectrophoresis (CIE) is a non-denaturing, 2-dimensional immunochemical technique known for its high resolution and specificity (Høiby and Axelsen, 1973; Smyth *et al*, 1978; Owen, 1985), and well-suited to study the immunogenicity of bacterial components (Lam *et al*, 1983). CIE has several advantages, in particular the ability to enable antigens to interact with their homologous antibody in a non denaturing system, thus allowing an assessment of native antigenic properties (Owen, 1985). The final result for a multicomponent antigen mixture reacted with hyperimmune sera is an impressive but complex array of immunoprecipitates as described by several workers (Høiby and Axelsen, 1973; Høiby, 1975; Lam *et al*, 1983). In these

systems many of the peaks have yet to be identified, but nonetheless, the technique has been successfully used to monitor the response to key antigens in *in vivo* infections.

In this study water soluble antigens from *P. aeruginosa* PAO-1 were prepared as the standard antigen reference system. The immunoprecipitin pattern obtained on reacting these with the reference antisera described (section 2.7.1) contained more than 30 precipitins. Although exact reproduction of the pattern described by Lam *et al* (1983) and Høiby and Axelsen (1973) was not achieved, there were no major differences in the antibodies produced against the more prominent antigens. Reasons for the apparent discrepancies may well include the different media and conditions used to prepare the reference antigens. The above researchers used cells cultivated under iron-sufficient conditions on the surface of solid Trüche agar at 35°C compared to the liquid CDM-Fe at 37°C used in this study. The bacterial cell surface is known to adapt to the environment in which it grows and hence the expression of bacterial antigens will be dependent on the culture conditions used (Brown and Williams, 1985 a and b). Furthermore, the nature of the antigens used to immunize the rabbits is important. Høiby and Axelsen (1973) used whole cell sonicates thus presenting more antigens to the immune system than the formalin-fixed cells used here, where the immune response will be largely towards those antigens preserved on the cell surface (Garvey *et al*, 1977).

In all these systems the reference antigens include only those which are water soluble after disruption of the cells by sonication and French press. Lam *et al* (1983) found that although OMP antigens were evident in the *P. aeruginosa* PAO-1 antigen preparation as shown by SDS-PAGE analysis, they were present only in extremely low quantities and were unlikely to be detected in CIE. This is hardly surprising since they are hydrophobic proteins embedded in the bacterial outer membrane, and hence complete

solubilization would require the presence of detergents (Owen, 1985). Detergent extraction of purified OMs using Triton X-100 (4%) in 50mM Tris HCl (pH8.6) and 5mM EDTA (Owen, 1985) was attempted in order to study OMPs in this system. However, Triton X-100 was found to slow down the electrophoretic movement of all antigens resulting in a clustered precipitin pattern with poor resolution. Similar observations were made by Lam (1983) with *P. aeruginosa* antigens. The use of individually purified OMPs in CIE as used by Lam *et al* (1983) or SDS-PAGE/CIE (Owen, 1985) or, alternatively, techniques such as immunoblotting may be more suitable for studying the antibody response to these OM antigens.

Despite these limitations, the sonicated whole cell preparations described by Lam *et al* (1983) and Høiby and Axelsen (1973) were acceptable as base line references to investigate antibody production to particular antigens.

The notably simplified precipitin patterns obtained with serum from rabbits in both models of infection emphasize that immunization with formalin-fixed whole cells elicits an immune response which does not entirely reflect that seen during an *in vivo* infection. In the former, animals are not subjected to the same physiological stress as occurs in an infection. The precipitin peaks observed in the rabbit models presumably represent those antigens which are sufficiently immunogenic to stimulate antibody formation, and in addition, are accessible to the cells of the immune system responsible for antibody formation.

In rabbits with disseminated peritonitis there was a marked and increasing response as time progressed to an antigen identified by crossed-line immunoelectrophoresis as LPS or an LPS/protein complex. The LPS of other Gram negative bacteria have a similar mobility and morphology in CIE (Schiøtz *et al*, 1979; Collins *et al*, 1983).

There appeared to be a notably poorer response to OM antigens by rabbits with biomaterial-associated infection. In the case of LPS, this was true even 8 weeks after implantation of the devices and hence at least 4 weeks after bacterial numbers in the order of 10^8 to 4 x 10^9 were found to be present on the disc surfaces. Such results may help to explain how a bacterial infection can be considered "cryptic" as suggested by Costerton (1984). Planktonic bacteria released in large numbers into the peritoneum during the disseminated peritonitis presumably expose many cell surface antigens, and also shed OM vesicles containing LPS. The spontaneous release of LPS by Gram-negative bacteria including *P. aeruginosa* PAO-1 in liquid media is well established (Devoe and Gilchrist, 1973; Lindsay *et al*, 1973; Pike and Chandler; 1974, Cadieux *et al*, 1983). If the immune system is intact it can rapidly respond and produce antibodies sufficient to overcome the infection. Additional defence mechanisms are operational in the peritoneal cavity and are efficient at dealing with an invasion of planktonic bacteria. Phagocytic cells are abundant such that in conjunction with the antibodies produced, bactericidal action is rapid (Vas, 1983). In addition, free-floating bacteria are quickly absorbed from the peritoneum for distribution to systemic defence systems and further stimulation of humoral immunity, so increasing the clearance efficiency (Skau *et al*, 1986). It is likely that these host defence factors all played a role in clearing the widespread infection in rabbits with the disseminated peritonitis. Only if bacterial numbers or endotoxin release were too great were the defences overwhelmed with fatal consequences, as shown by some rabbits.

It appears, however, that biofilm-enclosed bacteria colonizing the surface of implanted biomaterials were able to resist or evade the defence mechanisms described and continue to proliferate. It is known that although peritoneal macrophages are attracted to foreign biomaterials (Leake and Wright, 1979), opsonization is less effective since PMNs may degranulate

on contact with some foreign surfaces (Zimmerli *et al*, 1982). Certainly this could be inferred from the implanted devices in this second model of infection, as after 4 weeks, numerous white cells and macrophages were adjacent to the disc surfaces. However, the bacteria embedded in the glycocalyx remained viable and had continued to multiply. The immunological data suggests that, in addition to this factor, persistence of such bacteria may be due to their ability to grow and proliferate without a strong stimulation of the humoral immune system. It is probable that production of the fibrous glycocalyx matrix, which occurs very soon after initial adsorption of the bacteria on to the surface (Marshall *et al*, 1971), acts to mask key bacterial antigens such that they no longer induce extensive antibody formation. Consequently, the bacterial biofilm can become well established, probably also without causing any overt clinical symptoms. Certainly, many patients with heavily colonized prostheses experience few signs of infection (Costerton *et al*, 1985). At some stage it is possible that planktonic bacteria will be released and cause a more widespread peritonitis and the appearance of a strong antibody response may well coincide with such an episode. Indeed, in one rabbit with biomaterial-associated infection, antibodies to the LPS antigen were seen developing (Fig. 6.10, iv). Whether or not disseminated infection occurred at this stage was not established. Such antibodies may be effective at clearing the acute infection but not to destroy the glycocalyx-enclosed bacteria remaining within the biofilm.

Lam (1980) found a correlation between the intensity of antibody response, and bacterial counts in a rat model of chronic lung infection, and suggested that such immunological data could be used to provide predictive values of approximate numbers of bacteria harboured by a patient. This may indeed be possible in certain infectious states. However, the results of this investigation indicated that in patients with implanted prosthetic

devices who develop infection, information extrapolated from the immunological response may be misleading and result in an under estimate of the true numbers of bacteria colonizing the biomaterial, at least in the early stages.

The development of the Tenckhoff catheter (Tenckhoff and Schechter, 1968) composed of Silastc sheeting, has led to the increasing use of continuous ambulatory peritoneal dialysis (CAPD) as a means of managing patients with renal failure (Vas, 1983). Such patients are known to experience recurrent episodes of peritonitis which can severely limit the usefulness of this technique (Rubin *et al*, 1980; Fenton *et al*, 1981, Gokal, 1982). Peritonitis due to Gram-negative bacilli is usually more severe clinically than are episodes due to Gram-positive cocci and those caused by *P. aeruginosa* are known to be associated with a low cure rate unless the catheter is removed (Gokal, 1982; Krothapalli *et al*, 1982). SEM and TEM studies of the surfaces of Tenckhoff catheters removed from patients have confirmed the heavily colonized nature of these devices when they have been in place for over one month, and have indicated that the mode of growth of infecting bacteria is in the form of biofilm-encased adherent microcolonies (Marrie *et al*, 1983; Dasgupta *et al*, 1986). This study was clearly not attempting to mimic the clinical condition of CAPD patients. Nonetheless, the information gained may have some bearing on infections associated with these patients or indeed any other patients with indwelling prosthetic devices. It perhaps helps to explain how the biofilm bacteria become established on the prostheses without markedly stimulating humoral immune defences and, hence, how a nidus of infection is allowed to develop which may at any time initiate more widespread peritonitis attacks Important areas for future research may well involve steps to reduce initial biofilm formation on the indwelling catheters perhaps by using biomaterials resistant to their development. Alternatively reinforcement of

host defences to prevent dissemination of biofilm bacteria, or development of antibiotics which can successfully penetrate the protective biofilms, may have significant benefits.

Immunoblotting with serum taken sequentially from rabbits during the infection, in addition to affirming the CIE data, helped to determine which OM antigens were recognized by host immunoglobulins, and at what stage in the infection the antibodies were produced. In the disseminated peritonitis these antibodies were presumably important in overcoming the acute phase of the infection.

Using LPS-absorbed sera to maximize the response to OMPs, IRMPs and proteins D and E were shown to be among the first OMPs to be recognized. Serum IgG antibodies detected these antigens as early as 4 to 8 days after onset of disseminated infection again suggesting they may be important targets of host defence mechanisms. This response was further reflected locally by antibodies present in the pus or peritoneal fluid. Local immunity is clearly paramount in halting spread of the infection. The recognition of IRMPs by rabbits in both models of infection provides indirect evidence that bacteria were expressing these antigens during *in vivo* growth, hence implying that they were growing in an iron-limited environment in both circumstances.

As time progressed, antibodies to porin protein F developed, as observed in burn patients (see section 5.3), along with a marked response to H_1 and H_2. Of further interest was the strong recognition of the 55K antigen which co-electrophoresed with purified flagella protein despite there being insufficient protein present to be visualized with Coomassie blue stain or amido black stain. Its marked antigenicity has been observed by other workers (Mutharia *et al*, 1982; Anwar *et al*, 1984), and was also seen in burn patients (Section 5). Clearly bacterial flagella are important

for mobility, enabling planktonic bacteria released from the biofilm to migrate to and colonize other tissues, and hence spread the infection.

Overall then, the pattern of response to OMPs in the disseminated peritonitis model was similar to that seen in burn patients colonized with *P. aeruginosa* (Section 5), an infection considered to be acute in nature.

LPS was also observed to be immunogenic in the latter model stimulating the production of a high titre of antibodies. Both high molecular weight LPS molecules with long 'O' side chains, and rough LPS (core/lipid A) were detected by these antibodies suggesting that the latter are accessible to the immune system, and not totally shielded by the 'O' antigen polysaccharides. Wilkinson and Galbraith (1975) and Drewry *et al* (1975) have reported a relatively high proportion of R-form molecules lacking 'O' side chain material in some strains of *P. aeruginosa*, only 5 to 10% of core oligosaccharides carrying the 'O'-specific polymer. The potential of vaccines based on LPS and high molecular weight PS derived from LPS is discussed in section 1.5.

This study primarily focussed on the IgG response of the rabbits largely because these antibodies have been shown to offer better protection than IgM against experimental *P. aeruginosa* infections (Bjornson and Michael, 1970). Moreover, they have a longer half life in serum (23 days) compared to IgM (5.1 days), and finally, are known to penetrate secretions more effectively (Pennington, 1979). However, a study of the IgM response to OM antigens during the course of infection would be beneficial and should be considered for future work.

As a conclusion to this work, such immunological techniques enabled a study of the early immune responses to 2 types of infection, a disseminated peritonitis and a localized biomaterial-associated infection characterized by the presence of glycocalyx-enclosed bacterial biofilms. Hence,

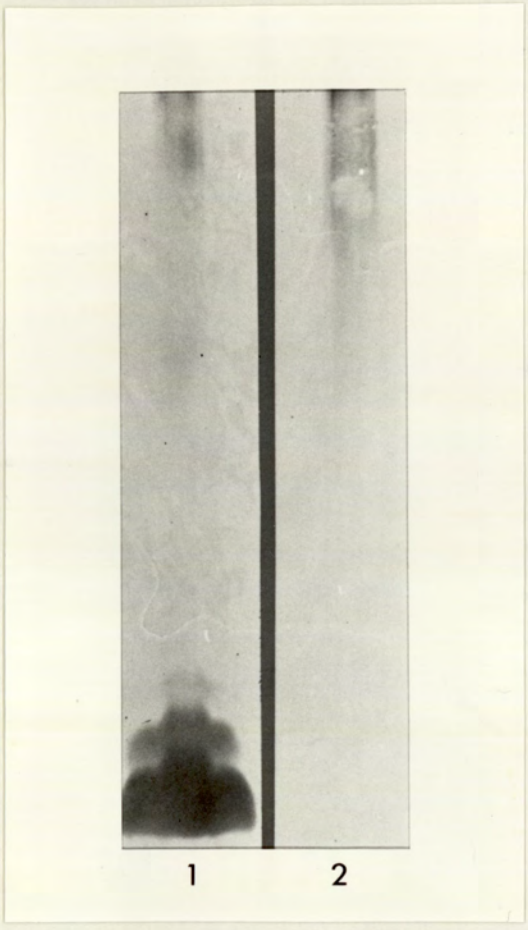
differences between the two models were monitored, and the key antigens recognized in both cases established. The information gained may help to formulate a more effective strategy for the future immunological control of such infections.

7. Studies on the immunogenicity and purification of *P. aeruginosa* IRMPs.

7.1. Immunogenicity of individual *P. aeruginosa* OMPs - raising of a monospecific polyclonal antisera.

The method of Knudsen (1984) (section 2.7.2) was used to produce individual OMP preparations for administration to mice to determine if they elicited an immune response. Briefly, OMPs were separated by SDS-PAGE following incubation in sample buffer at 37°C, electrophoretically transferred to NC paper and individual protein bands excised. Aitchison *et al* (1987) demonstrated that transfer of *Streptococcus faecalis* protein antigens to NC paper followed by incubation in 0.5M sodium periodate pH 4.0 for 1 h at 37°C resulted in oxidation of a 37K glycosylated protein so that it no longer reacted with hyperimmune rabbit sera. It was considered that similar treatment of OMPs of Gram-negative bacteria following transfer to NC paper may oxidise contaminating LPS on the blot, so destroying antigenic determinants on this molecule, or releasing it from the NC paper. Fig. 7.1,i, demonstrates probing of LPS immunoblots with rabbit antisera to LPS either with (lane 2) or without (lane 1) prior incubation of the NC strips in 0.5M sodium periodate. It appeared that sera raised against LPS reacted predominantly with lipid A/core molecules migrating to the bottom of the gel, with faint detection of higher molecular weight LPS not resolved into bands (Fig. 7.1,i; lane 1). After periodate treatment the rougher LPS was no longer recognized although high molecular weight material was still faintly visible. This suggests that sodium periodate may have oxidised antigenic determinants in the lipid A/core region, but did not destroy the '0' polysaccharide side chains. When OMP immunoblots were treated with sodium periodate prior to incubation in rabbit serum raised against *P. aeruginosa* whole cells (Fig. 7.1, ii) protein F was no longer detected

7.1, i.



7.1, ii.

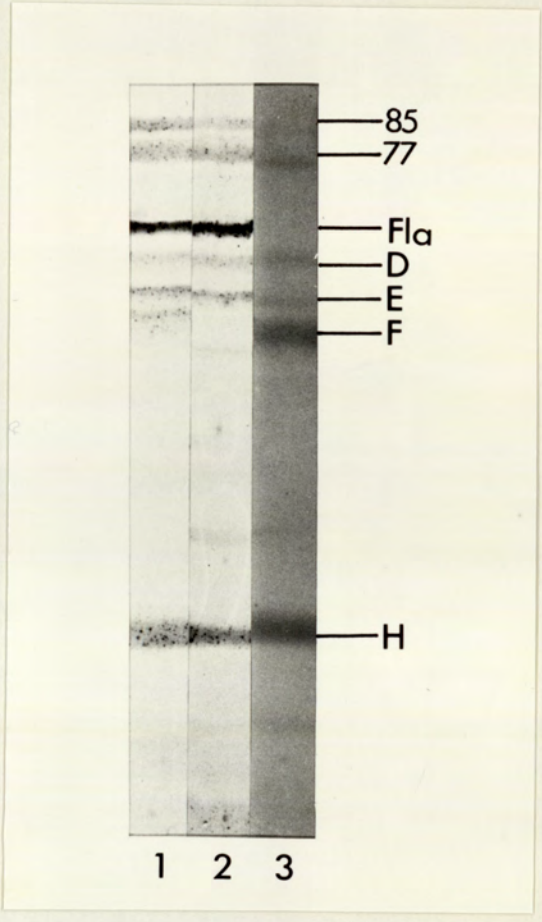


Fig. 7.1, i. Strip immunoblots of *P. aeruginosa* PAEW LPS probed with serum from a rabbit immunized with LPS. NC strip in lane 2 was pre-incubated in 0.5M sodium periodate for 1 h at 37°C.

Fig. 7.1, ii. Strip immunoblots of *P. aeruginosa* PAEW OMPs probed with serum from a rabbit immunized with whole cells of the same organism. NC strip in lane 2 was pre-incubated in 0.5M sodium periodate for 1 h at 37°C.

Lane 3 represents a replicate NC strip stained with amido black 1% w/v.

IgG antibodies detected with protein-A peroxidase.

(lane 2). It is possible that protein F contains glycosylated residues destroyed by sodium periodate so abolishing epitopes against which the antibodies were directed. However, there have been no reports indicating the glycosylated nature of this protein and the F protein cloned in *E. coli* retains its porin function (Woodruff *et al*, 1986) which suggests that it is not glycosylated. Alternatively, the results may reflect oxidation of rough LPS molecules co-migrating with the protein. The latter would indicate that visualization of protein F was indeed a reaction to LPS not the protein. Finally, it is possible that sodium periodate destoys amino groups and so may affect antigenic properties of proteins (Lugtenberg *et al*, 1983). Recognition of other OMPs was essentially unaffected by this treatment. It appeared, therefore, that incubation of proteins blotted onto NC paper with sodium periodate may help to reduce contamination with LPS.

Proteins F, H (H_1/ H_2) and the cluster of IRMPs were excised from NC sheets and half of the protein-bearing strips were treated with sodium periodate. These and the equivalent untreated strips were dried, macerated and dispersed in 500µl dimethylsulphoxide (DMSO), to dissolve the NC paper. Protein preparations were mixed with Alhydrogel (Centre for Applied Microbiology Research, Porton Down, Salisbury) as an adjuvant before inoculation into mice as described in section 2.7.2. Fig. 7.2 shows the results of probing OMP immunoblots with serum obtained from the immunized mice which indicate that the method was sucessful in raising a polyclonal antisera to the individual proteins (the collective group of IRMPs, F and H, Fig. 7.2, i, ii and iii respectively). In each case, on incubating immunoblots with serum from mice immunized with untreated protein, minor reactions with other antigens were faintly visible (lane 1), but these were barely detectable when probed with periodate-treated protein antisera (lane 2). This may suggest that LPS contaminating the untreated proteins may itself have elicited antibody formation and be responsible for the

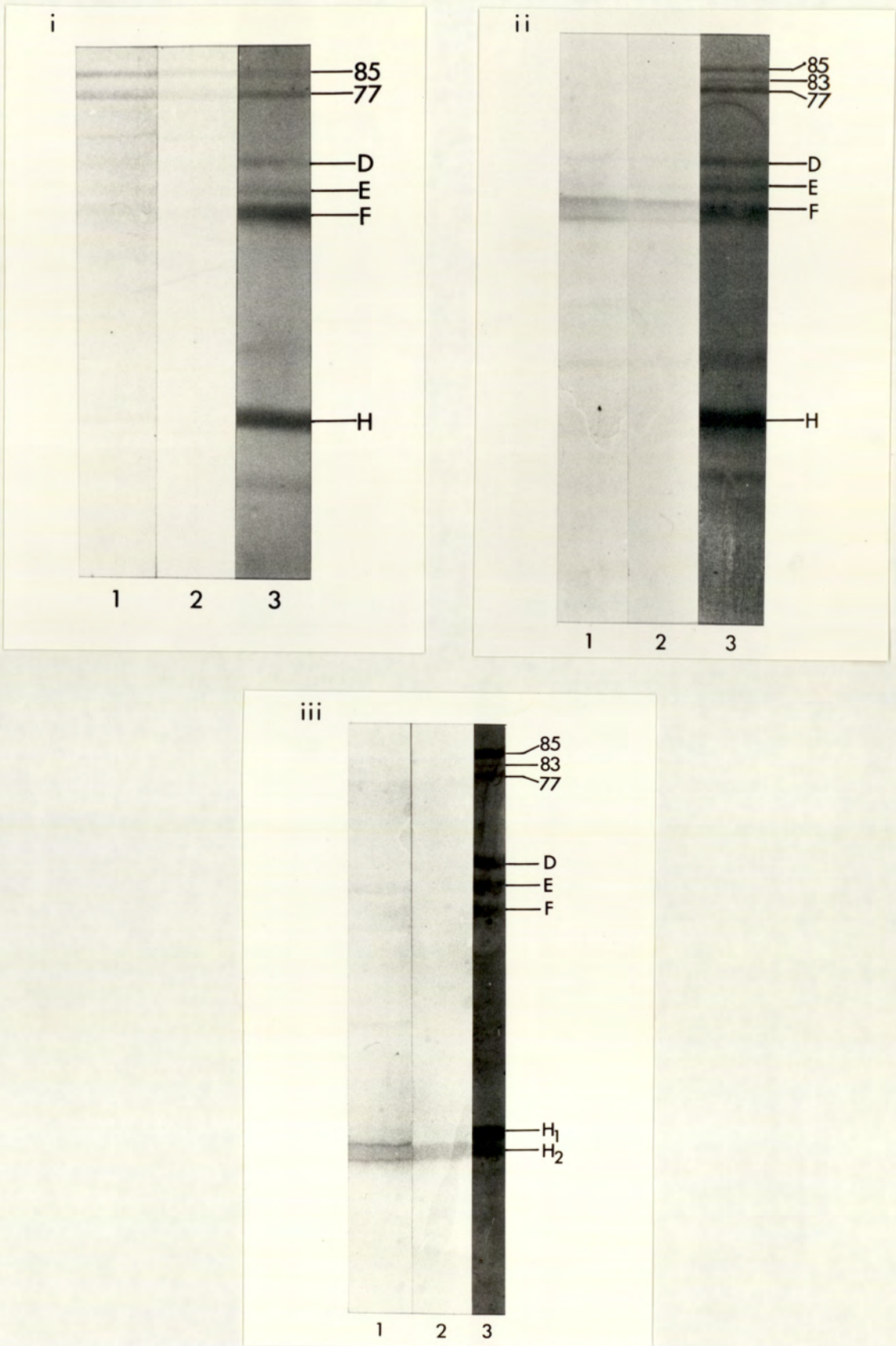


Fig. 7.2, i, ii and iii. Strip immunoblots of *P. aeruginosa* PAEW OMPs probed with serum from mice immunized with IRMPs (i), protein F (ii) or proteins H_1/H_2 (iii), excised from NC paper and dissolved in DMSO + Alhydrogel. Mice immunized with untreated protein-bearing strips (lane 1), or strips pre-incubated in 0.5M sodium periodate for 1 h at 37°C (lane 2).
IgG response detected with peroxidase-labelled anti-mouse IgG.
Lane 3 represents a replicate NC strip stained with amido black 1% w/v.

additional weaker bands detected. Furthermore, periodate treatment of the proteins may have destroyed some of the antigenic determinants of the LPS so resulting in an antisera more specific to the protein in question. Antibodies to the protein H_1/H_2 preparation appeared to be predominantly directed against protein H_2 (Fig. 7.2, iii)

A separate group of mice were immunized with periodate-treated protein H_1/H_2 in a similar manner using Freund's complete adjuvant (FCA) followed by Freund's incomplete adjuvant (FIA) (section 2.7.2) in place of Alhydrogel. Fig. 7.2, iv, demonstates that the FCA/FIA protocol was more effective at stimulating an immune response (lanes 1 and 2) than the Alhydrogel preparation (lanes 3 and 4). Finally, it was observed that a predominantly IgG response had been elicited (lanes 1 and 3) in both cases, with only a very low titre of IgM antibodies detectable (lanes 2 and 4).

Such a method may, therefore, prove useful for raising antisera to individual OMPs which could then be used passively to determine the protective nature of the antibodies so formed.

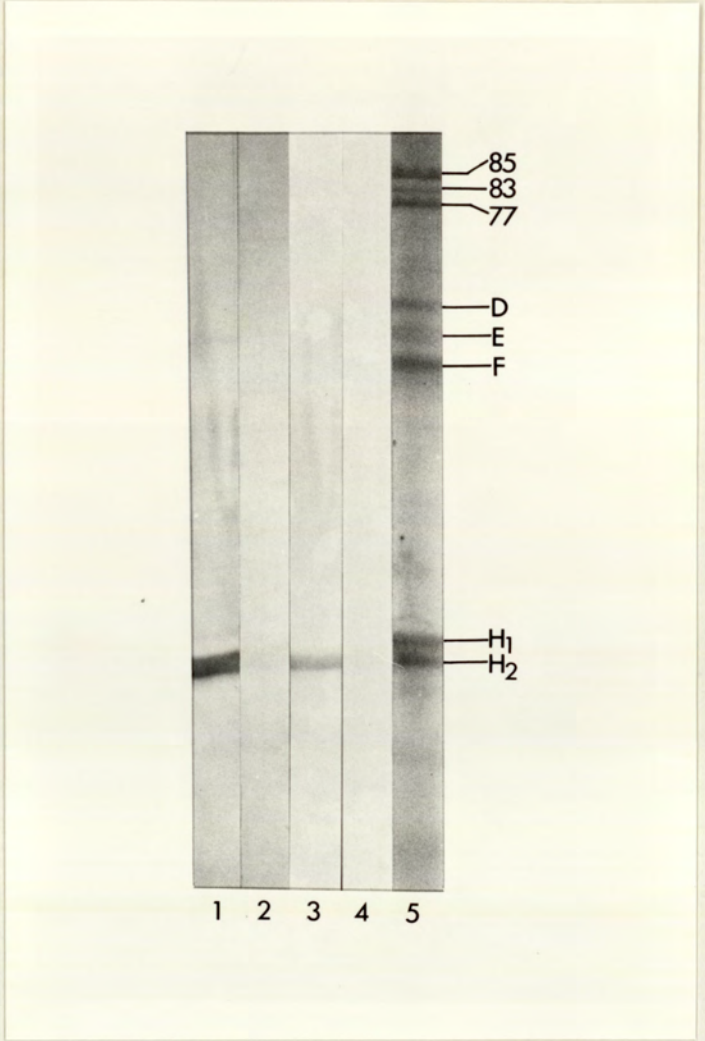


Fig. 7.2, iv. Strip immunoblots of *P. aeruginosa* PAEW OMPs probed with serum from mice immunized with periodate-treated protein H_1/H_2 plus Freund's complete and incomplete adjuvant (lanes 1 and 2) or Alhydrogel (lanes 3 and 4).

Reaction visualized with peroxidase-labelled anti-mouse IgG (lanes 1 and 3) or IgM (lanes 2 and 4).

Lane 5 represents a relicate NC strip stained with amido black 1% w/v.

7.2. Isolation of *P. aeruginosa* IRMPs

7.2.1. Solubilization of OMPs.

OMPs are embedded to a variable extent in the membrane bilayer composed of phospholipids, essentially present in the inner leaflet, and LPS distributed predominantly in the outer surfaces. Before any chromatographic procedures can be used to purify individual proteins the membrane has to be solubilized. This can be achieved in 3 ways: a) by mechanical means, b) by organic solvents and c) by detergents. Of these, detergent extraction is the most widely adopted procedure. A range of detergents may be employed which can be broadly classified as non-ionic, cationic or anionic by virtue of the charge on the detergent molecules (Helenius and Simons, 1975).

To investigate if any representatives of the 3 categories of detergents were effective in solubilizing the IRMPs, OM pellets from cells of *P. aeruginosa* PAEW cultivated in CDM-Fe were resuspended in a small volume of the detergent in question in 50mM Tris buffer pH 8.0. Preparations were incubated at room temperature for 1 h unless otherwise stated. Insoluble components were pelleted by centrifugation and the pellet resuspended to the same volume with water. Both the pellet (lane 1) and the supernatant containing the solubilized proteins (lane 2) were analyzed by SDS-PAGE as shown in Figs. 7.3 to 7.6. The non-ionic detergents tested (Fig. 7.3, i to v) were Tween 20, Tween 80, Triton X-100, Nonidet P 40 and N-octyl β-D glucoside. The mild agents Tween 20 and 80 (polyoxyethylene sorbitol esters) solubilized only a small fraction of the IRMPS, the majority remaining in the pellet. Low levels of flagella and proteins D, E, F and H were additionally present in the supernatant, but both detergents were less effective at the higher concentration of 5% (Fig. 7.3, i and ii, b). Triton X-100 (1%) and Nonidet P40 (1%), both polyoxyethylene p-t-octyl phenols, solubilized a greater proportion of IRMPs than the Tweens (Fig. 7.3, iii and iv), particularly in the presence of EDTA (Fig. 7.3, iiib and

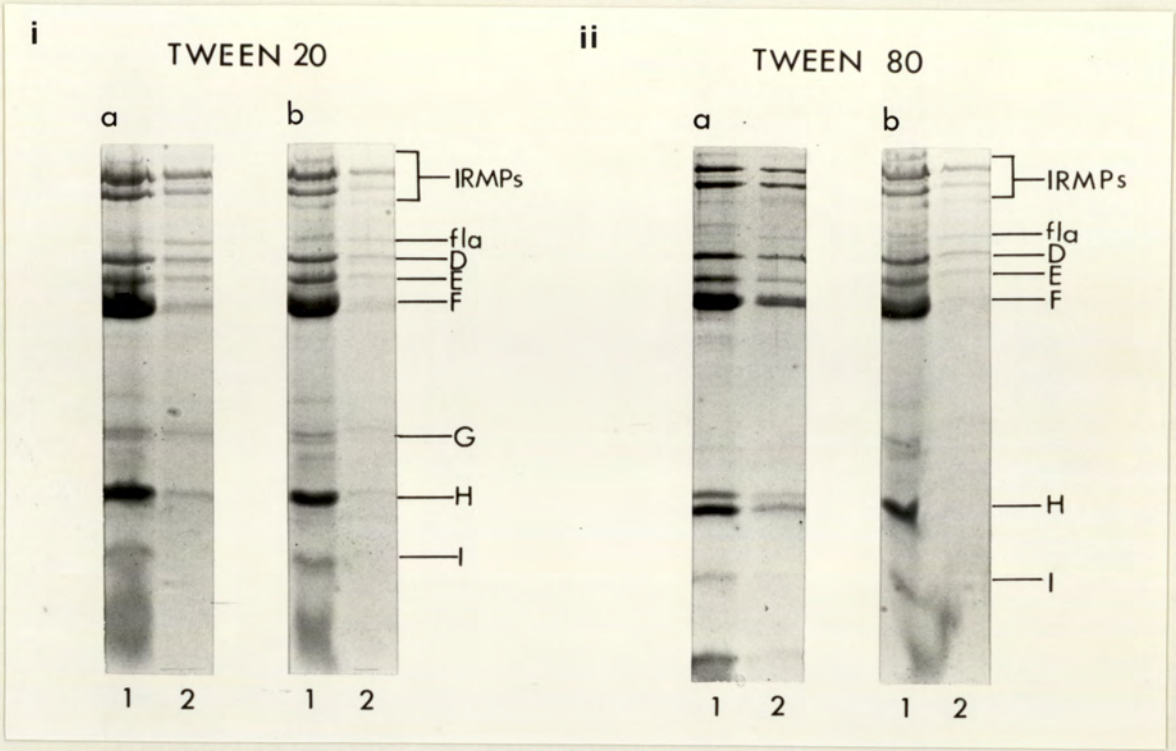


Fig. 7.3, i and ii. Soluble (lane 2) and insoluble (lane 1) fractions after extraction of *P. aeruginosa* PAEW OMs with non-ionic detergents as follows:
1% Tween 20 (i, a) 1% Tween 80 (ii, a)
5% Tween 20 (i, b) 5% Tween 80 (ii, b)

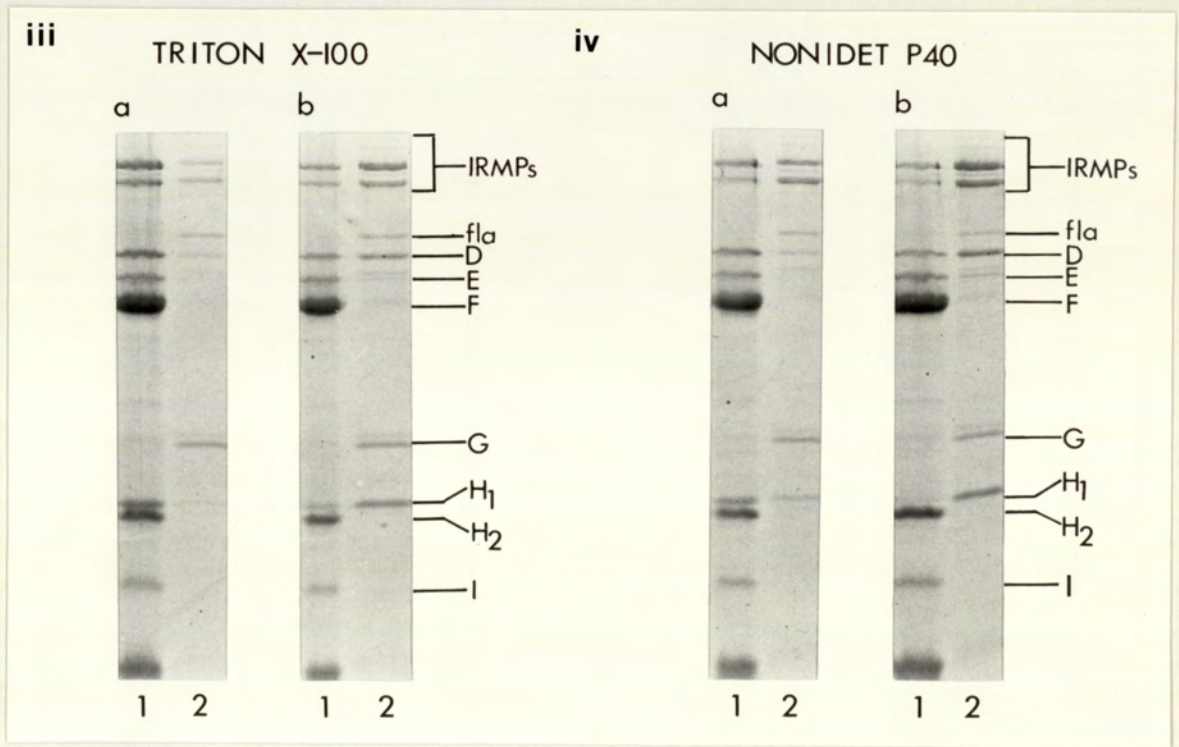


Fig. 7.3, iii and iv. Soluble (lane 2) and insoluble (lane 1) fractions after extraction of *P. aeruginosa* PAEW OMs with non-ionic detergents as follows:
1% Triton X-100 (iii, a) 1% Nonidet P40 (iv, a)
1% Triton X-100 + 10mM EDTA (iii, b) 1% Nonidet P40 + 10mM EDTA (iv, b).

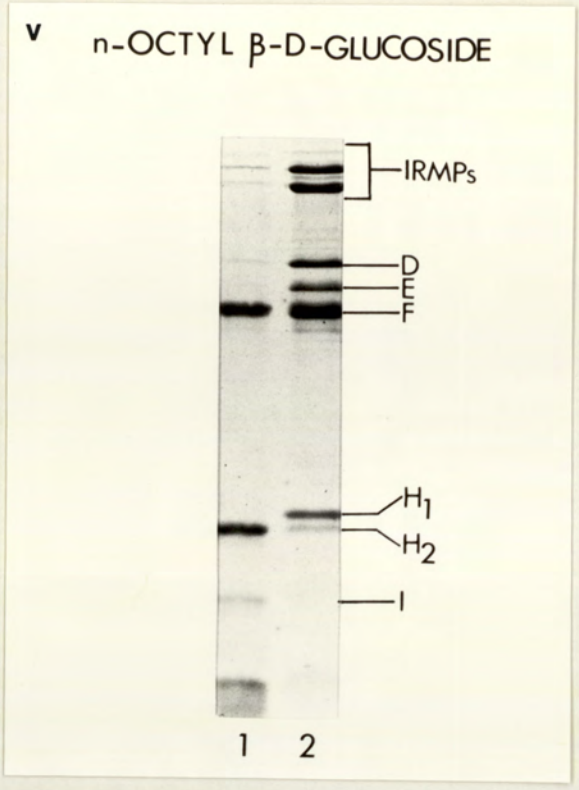


Fig. 7.3, v. Soluble (lane 2) and insoluble (lane 1) fractions after extraction of *P. aeruginosa* PAEW OMs with the non-ionic detergent N octyl β-D glucoside (1%).

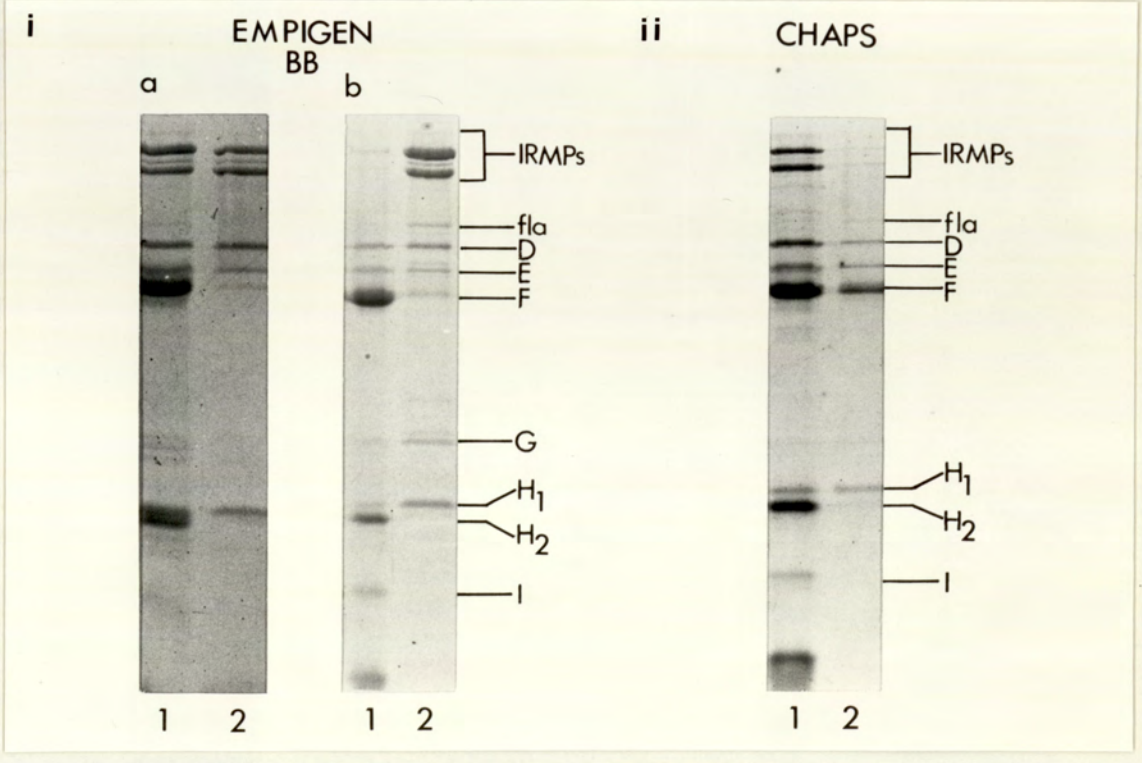


Fig. 7.4, i and ii. Soluble (lane 2) and insoluble (lane 1) fractions after extraction of *P. aeruginosa* PAEW OMs with zwitterionic detergents as follows:
0.3% Empigen BB (i, a) 1% CHAPS (ii)
0.3% Empigen BB + 10mM EDTA (i, b)

ivb). However, a substantial proportion of the IRMPs remained insoluble. Flagella, proteins D, E and G were also moderately soluble, and significantly more of protein H_1 was present in the supernatant when EDTA was included in the detergent solution. Proteins F, H_2 and I were not extracted. The latter are known to be peptidoglycan-associated proteins requiring elevated temperatures and SDS to remove them from the OM (Mizuno and Kageyama, 1979b; Hancock *et al*, 1981). Lastly, N octyl β-D glucoside 1% (Fig. 7.3, v) was found almost completely to solubilize the IRMPs along with proteins D, E and H_1, and a small amount of protein F.

Zwitterionic detergents included Empigen BB (an alkyl betaine) and CHAPS (3-[(3-cholamidopropyl) dimethylamino]-1-propane sulphonate). Empigen BB 0.3% was moderately effective on its own at solubilizing the IRMPs (Fig. 7.4 i, a), and showed more promise when EDTA was included (Fig. 7.4, i, b); the supernatant being considerably enriched with these high molecular weight proteins. Again, D, E and H were partly extracted. On the other hand, the majority of the IRMPs remained insoluble after extraction with 1% CHAPS with only a degree of solubilization of D, E, F and H_1 (Fig. 7.4, ii).

The cationic detergent cetyl trimethylammonium bromide (CTAB, 1%) was totally ineffective at solubilizing the IRMPs, only protein H_1 evident to any degree in the supernatant fraction (Fig. 7.5).

Finally, 2 anionic detergents were tested, sodium deoxycholate (Fig. 7.6, i), similar in structure to bile salts such as sodium cholate, and the strongly anionic SDS (Fig. 7.6, ii). Sodium deoxycholate 1.5% alone (7.6, ii a) or with EDTA (7.6, i b) was only modestly successful in extracting the IRMPs and was not selective. However, the desired proteins were almost totally soluble in 1% SDS, only the peptidoglycan-associated proteins remained largely in the pellet after extraction.

From these results, it appeared that none of the chosen detergents extracted solely the IRMPs under the conditions employed. However, soluble

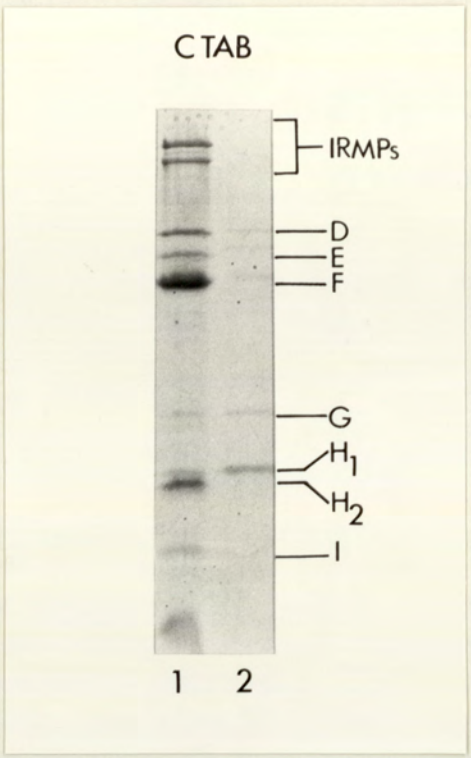


Fig. 7.5. Soluble (lane 2) and insoluble (lane 1) fractions after extraction of *P. aeruginosa* PAEW OMs with the cationic detergent CTAB (1%).

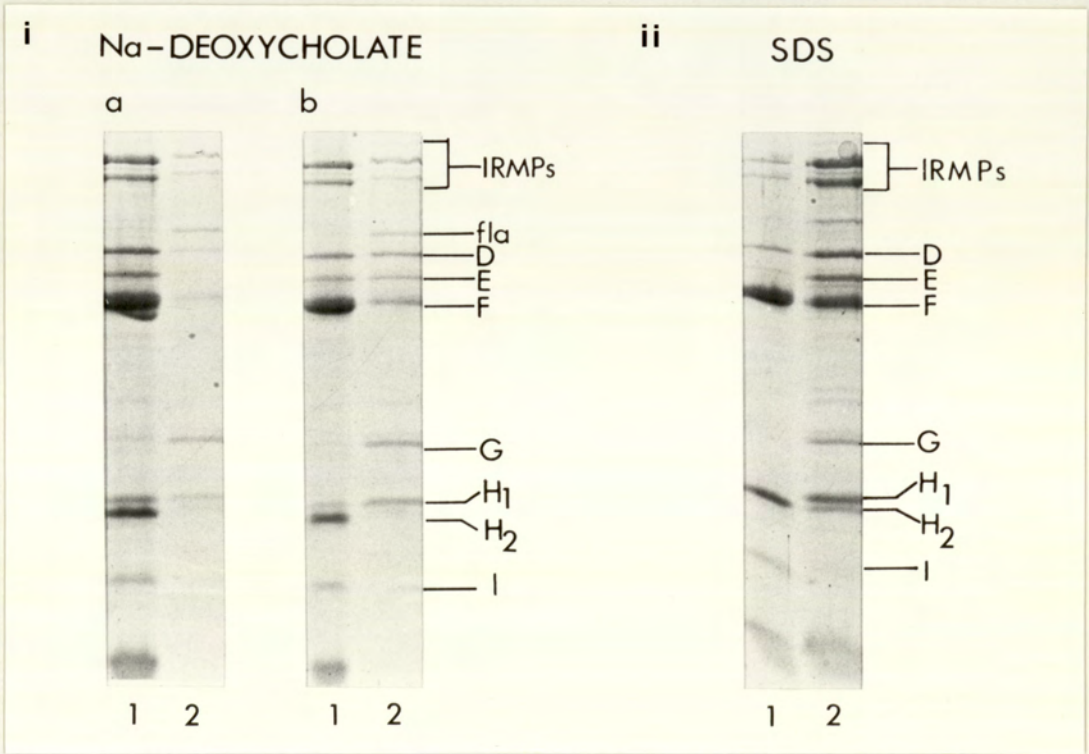


Fig. 7.6, i and ii. Soluble (lane 2) and insoluble fractions (lane 1) after extraction of *P. aeruginosa* PAEW OMs with anionic detergents as follows:
1.5% sodium deoxycholate (i, a) 1% SDS (ii)
1.5% sodium deoxycholate + 10mM EDTA (i, b)

fractions considerably enriched in the IRMPs were obtained with certain detergents, the most effective being N-octyl-β-D-glucoside, SDS and Empigen BB. Despite its high solubilizing power, further use of N-octyl-β-D glucoside was prohibited by its cost.

7.2.2. Gel filtration.

Having extracted IRMPs from the outer membrane in a soluble form, it was necessary to separate them from the other detergent-soluble OM proteins. Several chromatatographic procedures are available including gel filtration, ion-exchange chromatography and chromatofocusing, which rely on different properties to achieve separation.

Gel filtration, in which molecules elute in order of decreasing molecular size, was initially performed. Membrane proteins have a strong tendency to aggregate in solution, hence, to obtain optimum resolution, detergents were included in all buffers used during chromatographic procedures (Lambert, 1987).

OM pellets were extracted with SDS buffer (1% SDS in 50mM Tris HCl pH 8.0, containing 0.1M NaCl, 1mM phenylmethylsulphonylfluoride and 0.02% sodium azide) for 1 h at room temperature and the supernantant retained after centrifugation. Samples (200µl) were loaded onto a Superose HR 10/30 column equilibrated in the same buffer, and absorbance of the eluant monitored at 280nm. A typical UV absorbance trace is shown in Fig. 7.7,i, and 250µl fractions were collected as marked on the trace. These were lyophilized and analyzed by SDS-PAGE (Fig. 7.7,ii). Considerable separation had been achieved, fractions 3, 4, 5 and 6 containing the cluster of IRMPs relatively free of other contaminating OMPs. Clearly, later fractions also contained IRMPs along with lower molecular weight proteins. However, it was possible to collect these fractions, concentrate them by lyophilization and precipitate the proteins with 9 volumes of acetone at 4°C. The precipitated

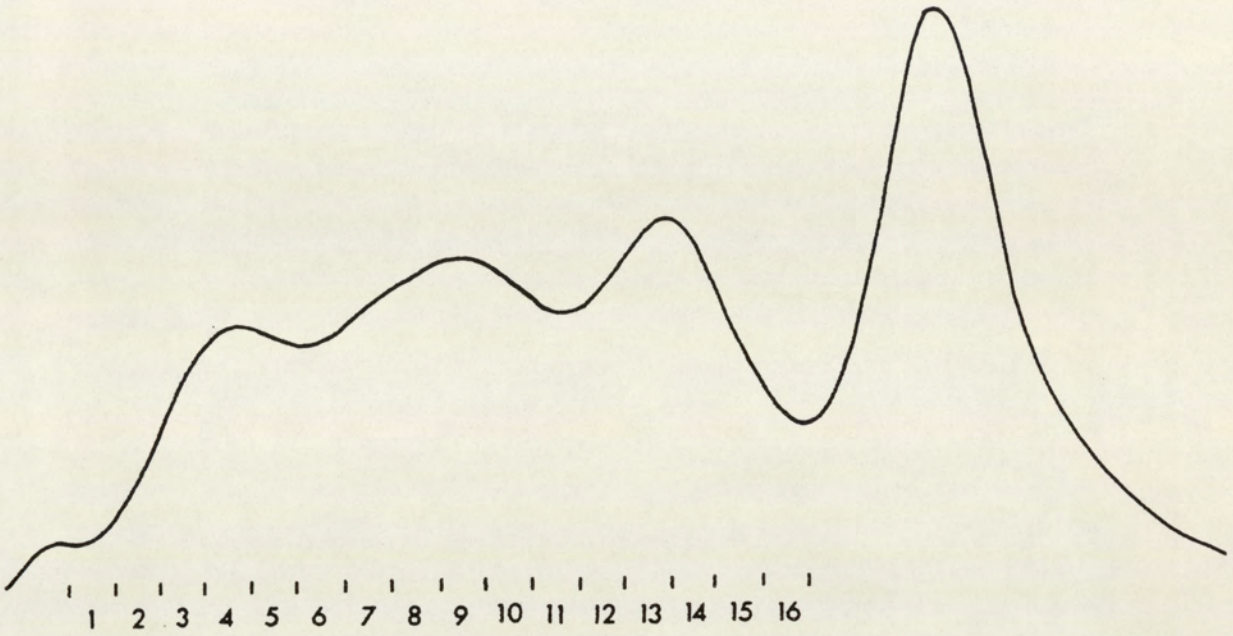


Fig. 7.7, i. UV trace obtained after gel filtration of 1% SDS extract (3·5 mg protein/ml). Column (Superose HR 10/30) equilibrated in 1% SDS buffer. Fractions 1 to 16 (250µl) collected as indicated.
Flow rate: 25ml/h.
Absorbance (280nm): 0.1

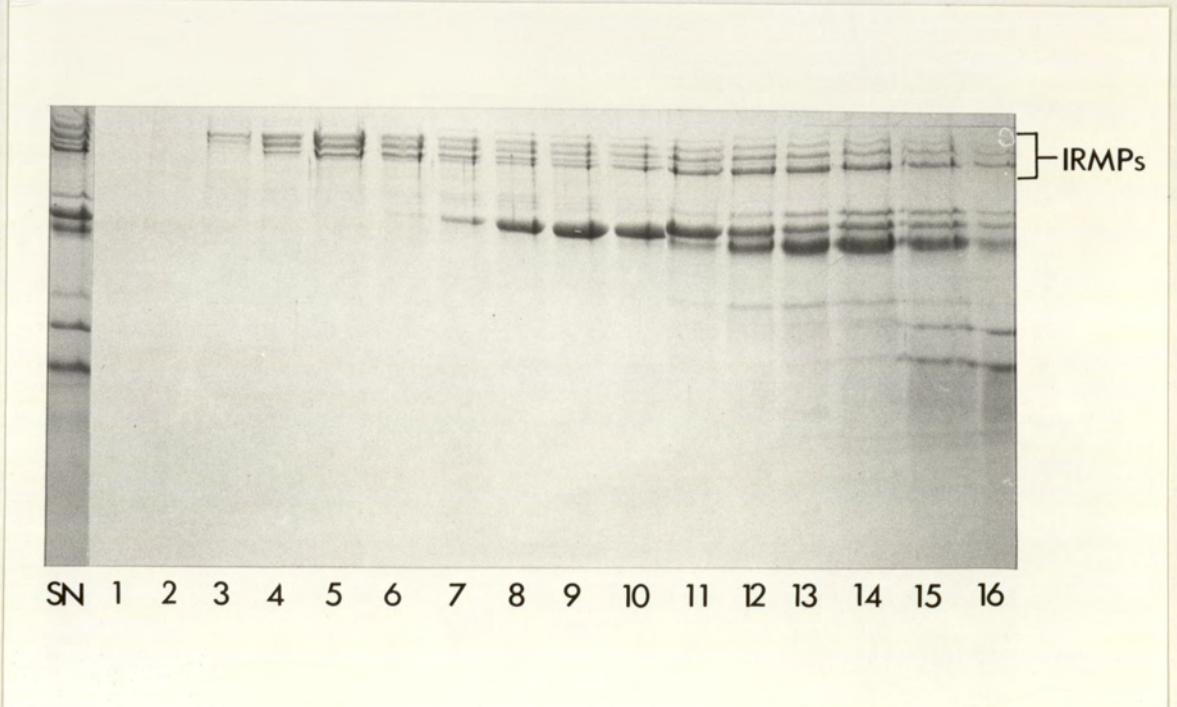


Fig. 7.7, ii. SDS-PAGE analysis of fractions 1 to 16 (lanes 1 to 16 respectively) collected after gel filtration as described above.
'SN' refers to 1% SDS extract (supernatant) loaded onto the column.

proteins were then resuspended in the buffer and applied to the column as before, thus increasing overall recovery of the desired antigens.

Gel filtration was repeated using OMs extracted with 0.1% SDS buffer and loaded onto the column equilibrated in the same 0.1% buffer. However, the degree of separation achieved was considerably reduced (Fig. 7.8, i and ii). The 1% SDS extract was additionally fractionated on the column equilibrated in 0.1% SDS buffer, but this resulted in even poorer resolution (Fig. 7.9, i and ii). These observations suggest that the higher concentrations of SDS were required to prevent the proteins from forming aggregates during chromatography.

Although the technique did not succeed in purifying individual IRMPs whose molecular weights do not differ by more than a few K, the cluster of high molecular weight proteins could be separated from the other major OMPs. Similar fractionation was achieved using 0.3% Empigen BB in place of 1% SDS in the extraction buffer and during gel filtration (data not shown). Repeated gel filtration runs using the 1% SDS extract enabled sufficient of the separated IRMPs to be collected, concentrated by lyophilization and then precipitated by addition of 9 volumes of acetone at 4°C, leaving the SDS in solution.

Proteinase-K digestion of these purified proteins followed by SDS-PAGE and silver staining revealed the familiar LPS ladder pattern (Fig. 7.10; lane 3) indicating that the proteins were still contaminated with this OM component. Several attempts were made to free the proteins of LPS. Parr *et al* (1986) described an electroelution method for purifying *P. aeruginosa* protein P following excision from SDS-PAGE gels. The crushed gel was placed in dialysis tubing containing 1% SDS and the tubing suspended in a Bio-Rad transblotting cell filled with transfer buffer. Electroelution at 50V for 5 h followed by 10V for 14 h was reported to result in a protein preparation devoid of detectable LPS. Similar electroelution was performed with the

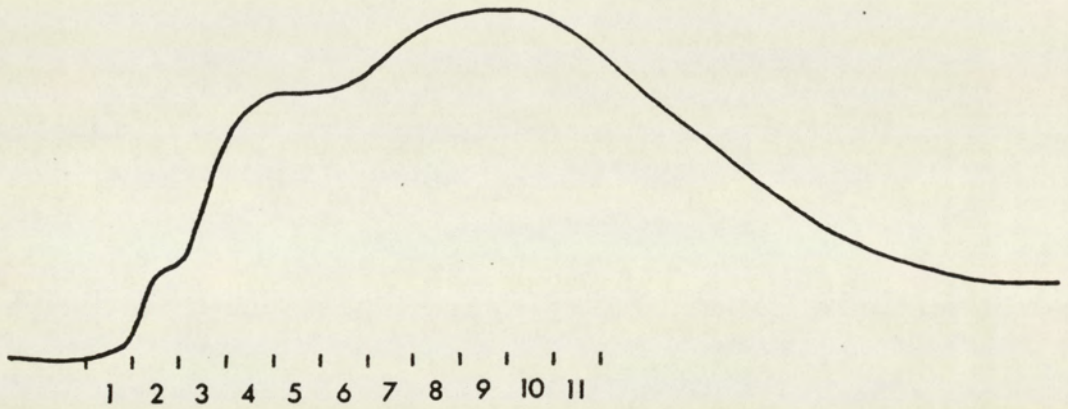


Fig. 7.8, i. UV trace obtained after gel filtration of 0.1% SDS extract (2 mg protein/ml). Column (Superose HR 10/30) equilibrated in 0.1% SDS buffer. Fractions 1 to 11 (250µl) collected as indicated.
Flow rate: 25ml/h.
Absorbance (280nm): 0.1.

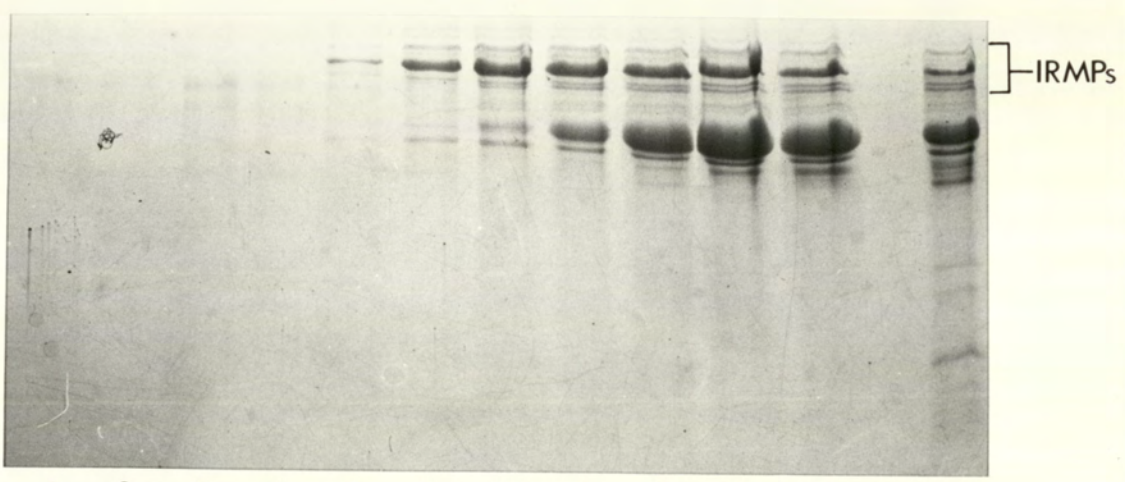


Fig. 7.8, ii. SDS-PAGE analysis of factions 1 to 11 (lanes 1 to 11 respectively) collected after gel filtration as described above.
'SN' refers to 0.1% SDS extract (supernatant) loaded onto the column.

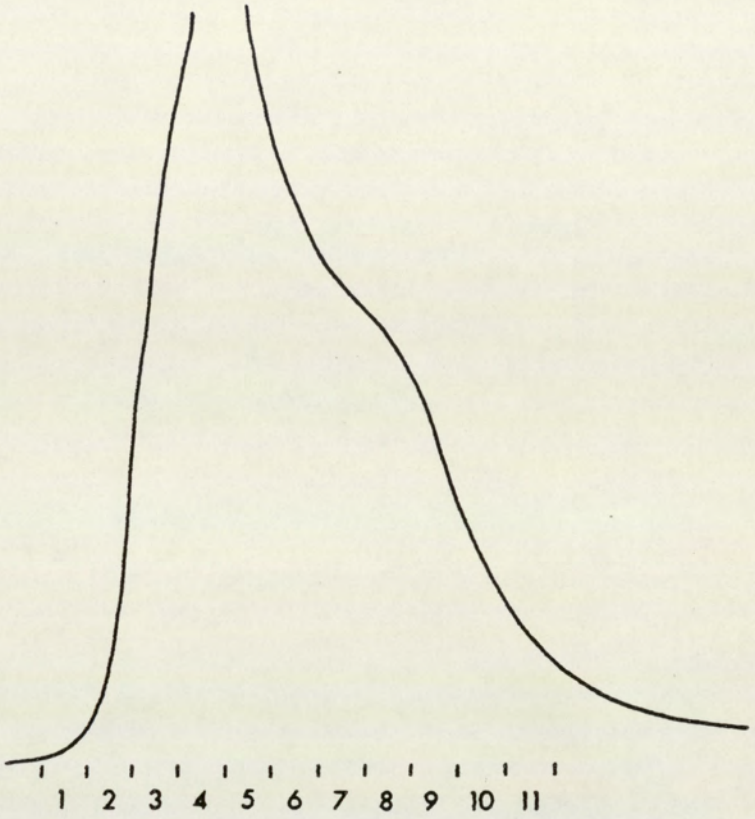


Fig. 7.9, i. UV trace obtained after gel filtration of 1% SDS extract (3·5 mg protein/ml). Column (Superose HR 10/30) equilibrated in 0.1% SDS buffer. Fractions 1 to 11 (250µl) collected as indicated.
Flow rate: 25ml/h.
Absorbance (280nm): 0.1.

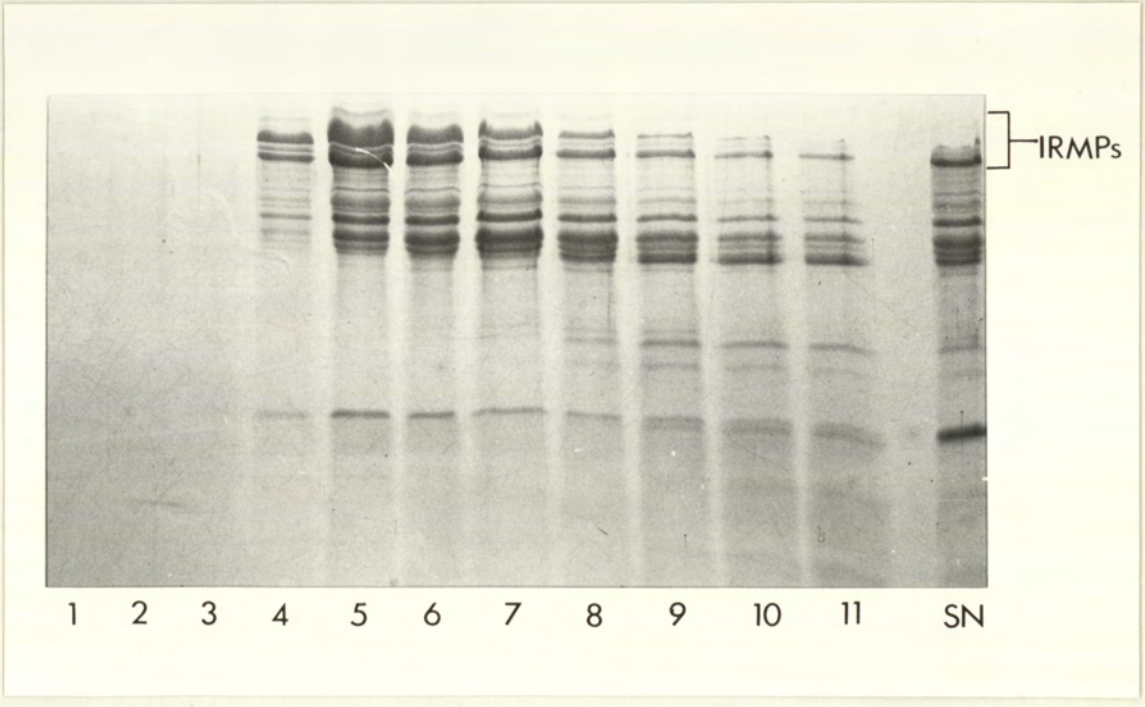


Fig. 7.9, ii. SDS-PAGE analysis of fractions 1 to 11 (lanes 1 to 11 respectively) collected after gel filtration as described above.
'SN' refers to 1% SDS extract (supernatant) loaded on to the column.

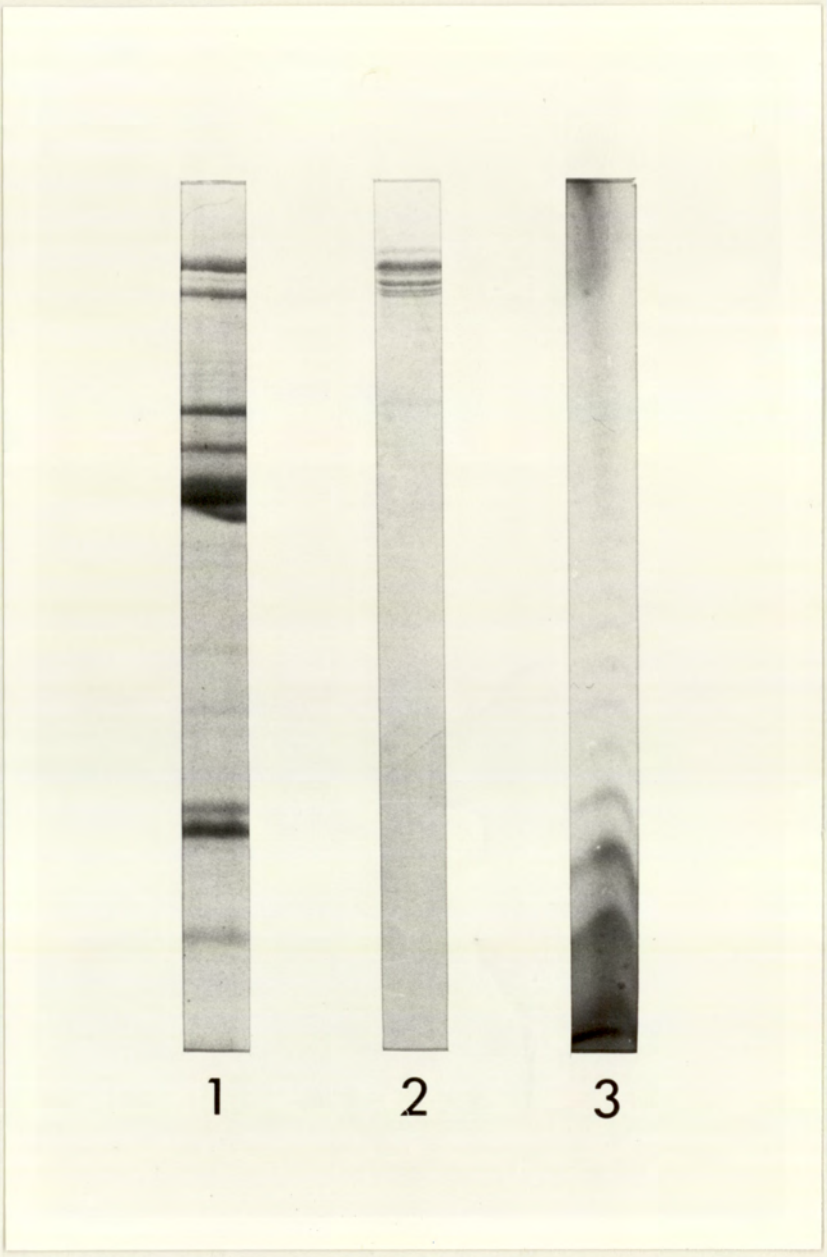


Fig. 7.10. SDS-PAGE analysis of *P. aeruginosa* PAEW OMs (lane 1), purified IRMPs (lane 2) and proteinase-K digests of the purified IRMPs (lane 3). Gels in lanes 1 and 2 were stained with Coomassie blue to detect protein. Gel in lane 3 was stained with silver to detect LPS.

purified IRMPs. However, little reduction in LPS contamination was achieved (results not shown).

An affinity chromatography medium for removing endotoxin (Detoxi-gel: Pierce Chemical Co. Illinois U.S.A.) claimed by the manufacturers to be effective at reducing LPS levels in protein solutions, was then tested according to the instructions supplied. However, both LPS and IRMPs bound to the ligand, the proteins remaining attached even after the buffer salt concentration was increased to 0.5M (results not shown).

Finally, Heckels (1977) described extraction of OMs from *Neisseria gonnorrhoeae* with 1% sodium cholate in a glycine buffer followed by gel filtration. 2 separate peaks eluted from the column, the first containing protein and the second LPS. Gnehm *et al* (1985) also reported chromatographic separation of *Haemophilus influenzae* LPS from OM proteins following solubilization in a similar LPS disaggregating buffer containing sodium deoxycholate. The purified IRMPs were therefore, suspended in 0.1M glycine/NaOH buffer pH9.5 containing 5mM EDTA and 1.5% sodium deoxycholate and loaded onto the Superose 12HR 10/30 column equilibrated in the same buffer. Complete solubilization in this buffer was achieved by brief alkalinization to pH 11.0 (Gnehm *et al*, 1985). Fractions (300µl) were collected, divided into 2 parts and lyophilized. One half of each fraction was analyzed for protein by SDS-PAGE and Coomassie blue stain, the other half analyzed for LPS by proteinase-K digestion, SDS-PAGE and silver stain as shown in Fig. 7.11. The IRMPs were found to elute in fractions 2 to 12 (7.11, i), whereas the LPS eluted in later fractions (11 to 22, Fig. 7.11, ii) and had itself been fractionated according to molecular weight. The presence of deoxycholate in the samples gave the IRMPs a rather wavy appearance on SDS-PAGE (Fig. 7.11, i) and resulted in a darker region at the top of the gel on silver staining (Fig. 7.11, ii). Nevertheless, the results indicate that this procedure may be useful in removing or considerably

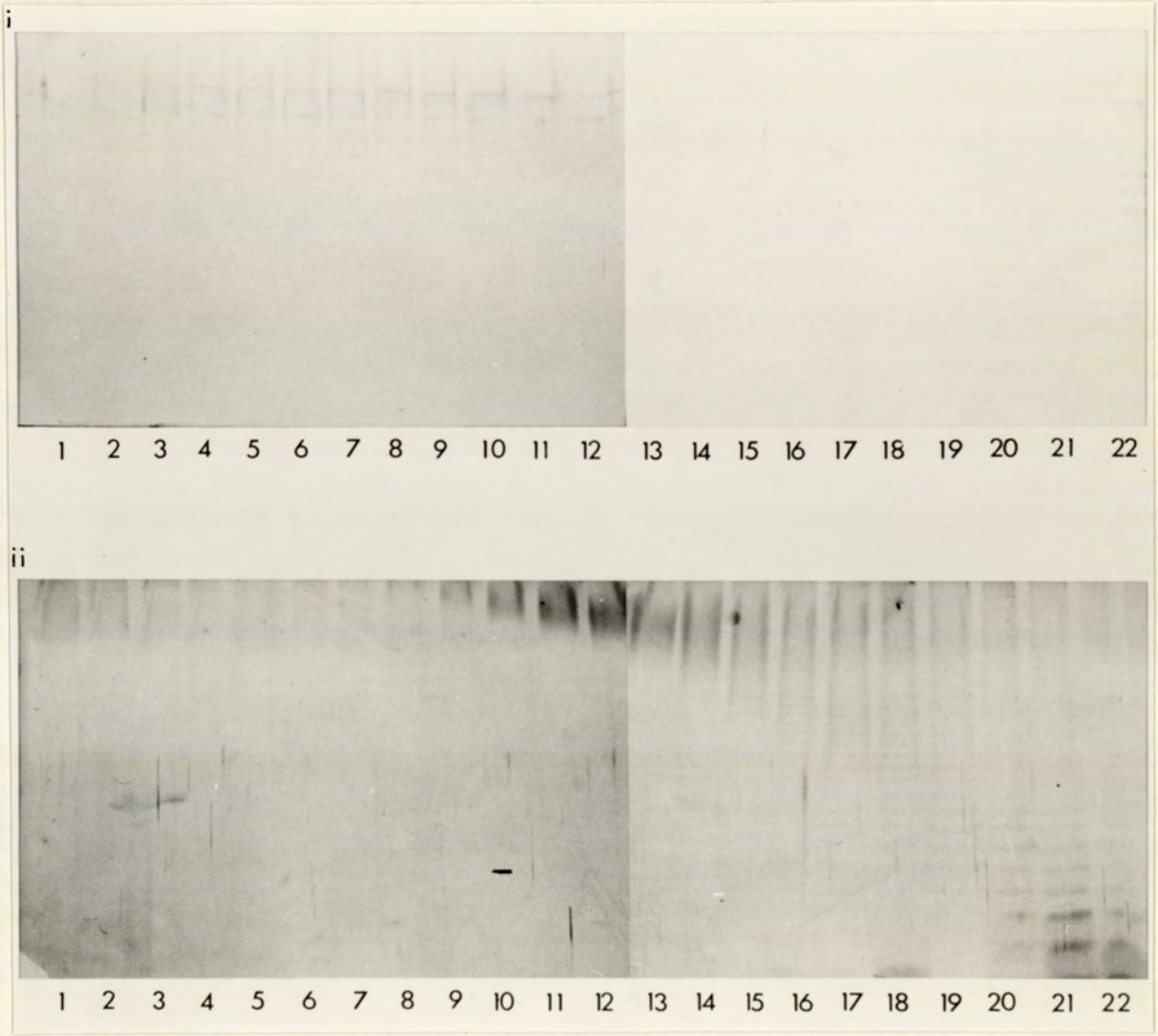


Fig. 7.11, i. SDS-PAGE analysis of fractions 1 to 22 (300µl) collected after gel filtration of IRMPs dissolved in 1.5% sodium deoxycholate buffer. (UV trace not obtained as sodium deoxycholate absorbs at 280nm). Column equilibrated in the same buffer.
Flow rate 25 ml/h.
Gel stained with Coomassie brilliant blue to detect protein.

Fig. 7.11, ii. SDS-PAGE analysis of fractions 1 to 22 collected after gel filtration of IRMPs as described above and digested with proteinase-K. Gel stained with silver to detect LPS.

reducing contaminating LPS in membrane protein preparations. Furthermore, silver-staining of proteinase-K digested samples separated on a mini-gel system provided a sensitive method for determining LPS levels in these samples. As little as 25ng LPS could be detected by this technique (data not shown) hence, it is far more sensitive than the KDO assay frequently used as a criterion for estimating LPS.

Sodium deoxycholate could be removed from the purified protein preparations by exhaustive dialysis at 4°C against 0.025M glycine pH 9.0. The high pH is required to prevent precipitation of sodium deoxycholate due to bile acid formation (Helenius and Simons, 1975). The proteins could subsequently be concentrated by lyophilization.

7.3. Discussion

Excision of protein bands from NC paper following electrophoretic transfer, based on the method of Knudsen (1984) provides a useful, method for raising a polyclonal antisera to a single protein or group of proteins as desired. Several researchers have prepared proteins directly from SDS-PAGE gels (Hjerten *et al*, 1969; Kabir, 1980; Ofverstedt *et al*, 1983; Gilleland *et al*, 1984; Parr *et al*, 1986). However, transfer to NC paper has the advantage that the proteins are freed from acrylamide and denaturing SDS and may in part regain their native formation (Hjerten, 1983). Incubation in sample buffer at room temperature prior to electrophoresis may also reduce the degree of denaturation. If a lower percentage of acrylamide was used in the initial separation it may be possible to excise the 2 major IRMPs (77 and 85K) individually.

A complication of using this method for Gram-negative organisms is contamination of the proteins on the NC paper with LPS (Poxton *et al*, 1985). LPS itself is immunogenic and elicits antibody formation, hence, if the antisera is subsequently used in passive immunotherapy studies any protection afforded may be due to anti-LPS antibodies. It is possible to overcome this in part by immunoprecipitating these antibodies after addition of LPS extracted from the same organism. In addition, a different *Pseudomonas* serotype may be used as the challenging bacteria. However, steps to reduce the level of LPS contamination are clearly desirable. It appeared that incubation of the NC strips in 0.5M sodium periodate may help to a degree in this respect, perhaps oxidising and thus destroying antigenic determinants on the LPS molecule, particularly rougher LPS.

The method demonstrated that the OMPs could stimulate the formation of antibodies which, furthermore, were of the IgG subclass. As discussed in section 6.6, IgG antibodies offer better protection against experimental

Pseudomonas infections than IgM (Bjornson and Michael, 1970), and have a longer half life (Pennington, 1979).

The superior adjuvant activity of FCA/FCIA compared to Alhydrogel was noted. Furthermore, addition of the water-based Alhydrogel encouraged precipitation of the dissolved NC paper unless the volume of DMSO was increased. This made incorporation of sufficient protein into a volume suitable for subcutaneous injection into mice more difficult. The mechanism of adjuvant action is complex. Components such as aluminium hydroxide and oil emulsions are thought to act primarily by sequestering the antigen so maintaining it at the inoculation site and allowing a slow release into the blood and lymphocyte circulation (Chedid, 1985). They may also modify antigenic properties so enhancing presentation of the antigen to cells responsible for humoral antibody production, or may act directly on cells of the immune system activating macrophages, B and T cells (Jollès and Werner, 1981). FCA contains whole mycobacterial cells and its potent adjuvant activity has long been recognized (Freund *et al*, 1937). However, undesirable side effects including lung granuloma, lymphoid hyperplasia (Ogonuki *et al*, 1966) and enhanced host sensitivity to endotoxin have prohibited its use in humans. Nevertheless, it can be used to good effect for raising antisera in animals. Alhydrogel is an aluminium hydroxide gel thought to increase the level of antibody production but not to stimulate delayed hypersensitivity (Jollès and Werner, 1981). Currently it is one of the main adjuvants permissible in human and veterinay medicine, hence its use in this study.

Having established that IRMPs are expressed by *P. aeruginosa* in situ in human burn wounds, are recognized by host antibodies and, that the separated proteins elicit antibody formation in mice it was desirable to develop a method to purify the proteins in larger quantities and in a more suitable form. Such proteins could then be tested in protection studies to

determine their value as vaccine candidates, or be used for raising protective antisera for passive immunotherapy. In addition, monoclonal antibodies could be produced to the purified proteins which themselves may have a role in passive immunotherapy, or in improved purification techniques, and surface localization studies. Finally, amino acid sequencing of the proteins could be performed which may aid in determining genetic control of their expression.

The initial step in purifying the IRMPs was to extract them from the OM in a soluble form. No detergents selectively solubilized the IRMPs without other OMPs, but soluble fractions highly enriched in the IRMPs were obtained with N-octyl β-D-glucoside, SDS and Empigen BB. Use of N-octyl β-D-glucoside was limited by its cost, but subsequent gel filtration of the crude mixture of SDS-soluble proteins yielded fractions containing the cluster of IRMPs free from other contaminating OMPs. Such a purification procedure was originally proposed by Lambert (1987). SDS is a strongly anionic detergent. Its binding to proteins is described as cooperative and is frequently accompanied by denaturation (Helenius and Simons, 1975). However, proteins differ in their intrinsic stability towards dodecyl sulphate denaturation and some proteins do not bind the detergent in a cooperative fashion with unfolding unless heat is applied (Laver, 1963; Scandella and Kornberg, 1971). Despite its denaturing tendencies, SDS has been widely used to purify bacterial membrane proteins due to its excellent solubilizing properties. Yoshimura *et al* (1983) purified *P. aeruginosa* protein F using gel filtration with dodecyl sulphate (lithium or sodium) and demonstrated that the protein retained its porin function and was as active as the native porin in OM fragments. Bub *et al* (1980) purified a 36K protein from *P. mirabilis* by a procedure involving solubilization at room temperature in 1% SDS. They demonstrated that the isolated protein reacted with antisera to *P. mirabilis* cell walls so suggesting that the protein

essentially retained its native configuration. The major OMP of *Chlamydia trachomatis* was purified with 2% SDS followed by gel filtration in 0.1% SDS and the authors claim that the protein retained at least some of its native antigenic properties (Caldwell *et al*, 1981). Finally, Munson and Granoff (1985) found that protein P5 from *H. influenzae* type b was solubilized in SDS at room temperature in its non-denatured form. If subsequent studies indicate significant denaturation of IRMPs following purification in SDS, the milder zwitterionic detergent Empigen BB could be substituted, similar separation being achieved with this detergent. Zwitterionic detergents are amphoteric surfactants which effectively solubilize membrane proteins without major loss of biological activity (Gonenne and Ernst, 1978). They have been succesfully used to purify several bacterial membrane proteins (James and Heckels, 1981; Blake and Gotschlich, 1982). A more detailed review on bacterial OM purification methods has been made by Lambert (1987).

Gel filtration was first introduced by Porath and Flodin (1959) and has subsequently been developed and extended by improving the gels to give faster processing and to cover a larger range of molecular sizes (Scopes, 1982). Pharmacia have recently developed the FPLC (fast protein liquid chromatography) system specifically for the separation of biomolecules including proteins. The Superose 12 HR 10/30 column is a gel filtration column containing a cross-linked agarose based medium with optimum molecular weight separation in the range $1x10^3$ to $3x10^5$. The narrow particle size distribution (average size 10µm + or - 2) of Superose enabled high flow rates at low back pressures and average separation times of between 30 and 45 min were achieved. In addition, the column and FPLC system as a whole were compatible with a wide range of detergents including SDS. Good separation of the IRMPs from the crude protein mixture was obtained with this column and, in addition, a second gel filtration step

performed with sodium deoxycholate buffer (1.5%) further purified the proteins from contaminating LPS.

Sodium deoxycholate is a non protein-denaturing detergent similar in structure to the bile salts, and is thought to disperse LPS aggregates into monomeric units (Ribi *et al*, 1966). Such units are lower in molecular weight than many OMPs hence enabling separation of the proteins from LPS by chromatographic procedures (Heckels, 1977). Shands and Chun (1980) determined molecular weights of LPS monomers of *Salmonella typhimurium* following dissociation of LPS aggregates with deoxycholate and obtained values of 5.5, 10.6, and 16.5K for Re, Ra and 'smooth' LPS respectively. They suggest that probable binding sites of the detergent include the fatty acids of lipid A , the phosphorylethanolamine groups in the deep core region and the amino group of N-acetyl glucosamine in the core regions. Inclusion of EDTA in the buffer assisted in disaggregating the LPS by chelating cations (Ca^{2+} and Mg^{2+}) important in maintaining integrity of the molecules. It is thought that the cations act as counter ions for the phosphate groups in the lipid A and phosphate and carboxyl groups in the core polysaccharide, reducing repulsive charges between LPS subunits and allowing the molecules to aggregate. Their removal would, therefore, increase the net negative charge and disperse the LPS by charge-charge repulsion (Shands and Chun, 1980). Gel filtration further fractionated the LPS according to molecular size, those with long 'O' side chains eluting first. The IRMPs largely eluted before the LPS but the last 2 or 3 IRMP fractions also contained the high molecular weight LPS material. Hence for future purification studies it may be worth selecting a rough mutant strain of *P. aeruginosa* lacking the smooth LPS molecules with long 'O' side chains.

In conclusion, the methods described have potential for purifying the collective group of IRMPs relatively free from LPS. It is possible that

other techniques such as ion exchange, affinity-adsorption or protein precipitation with high or low salt concentrations may further improve yields of the proteins or may succeed in resolving the individual IRMPs and should be considered for future work. Furthermore, if the genes encoding expression of the IRMPs are discovered, recombinant DNA techniques may enable insertion of the genes into plasmid carriers and introduction into other non-pathogenic bacterial cells. This would result in over expression of the proteins by these cells so overcoming the problems associated with large scale culturing of iron-limited bacteria.

8. Concluding Remarks.

With the ongoing desire to improve existing immunoprophylactic measures against infection empirical approaches to vaccine development have been largely superceded by a more rational mode of design. Thus, researchers have sought to produce preparations enriched in key bacterial antigens and from which non-essential, potentially toxic components have been eliminated. Continuing advances in the field of recombinant DNA genetics and hybridoma technology and a greater understanding of the molecular biology of many pathogens may lead to a new generation of vaccines in the near future composed of highly purified, specific antigens or even fragments of antigens. Alternatively, more effective passive immunotherapy regimes may be introduced, perhaps involving monoclonal antibodies directed against target antigens.

Crucial to the future success of any immunotherapeutic strategies is the identification of key bacterial antigens which will stimulate prolonged levels of protective immunity against infection. An important factor in this respect is the realization that bacteria may possess markedly different characteristics *in vivo* from those observed *in vitro*, some of which may correlate with increased virulence and pathogenicity of the organism (Brown and Williams, 1985 a and b). Researchers aiming to select alternative bacterial components, either cellular or extracellular, for incorporation into vaccines should not simply consider those expressed by the bacteria following growth in nutrient-rich liquid media. By adopting the latter approach valuable antigens or determinants of virulence important in infection may be missed altogether. Hence, an increased understanding of bacterial properties in the *in vivo* environment is essential.

To this end, an attempt was made to study OM antigens of bacteria growing *in situ* in human infection. OM components were selected since they mediate the interaction of the microorganism with its environment and are more likely to stimulate host immune defences. *P. aeruginosa* was recovered directly without subculture from the infected wounds of two patients with burn injuries and the OM antigens examined by SDS-PAGE. This technique provided an excellent means of separating and characterizing bacterial components by molecular weight and revealed important differences between the antigens of *in vivo* cells and those of the same isolate cultivated *in vitro*. A group of high molecular weight proteins were evident in the OMs of the former cells which were not expressed by bacteria grown in complex media unless the iron content of the media was reduced. These IRMPs have not yet been fully characterized in terms of their function but some, at least, are thought to act as receptors for iron-siderophore complexes so enabling bacterial uptake of this essential metal ion. Their expression by bacteria growing in burn wounds implies the iron-restricted nature of the wound site. Notable differences in the LPS profiles of *in vivo* and *in vitro* grown cells were also observed following SDS-PAGE analysis which may reflect biochemical differences in LPS composition, emphasizing once more the influence of the environment on such structures.

The question which subsequently arose was "which of the OM antigens expressed *in vivo* would constitute effective vaccine components?". Several vaccines based on LPS already exist and whilst they do induce a degree of protection against infection, their inherent toxicity and serotype specificty have precluded their widespread clinical use (Pennington, 1979). Hence, this study focussed largely on the OMPs which may provide "common" antigens of negligible toxicity. In an attempt further to answer the question an indirect approach was adopted whereby the host immune response to invading microorganisms during infection was studied and the bacterial

OMPs which evoked antibody formation determined. Immunoblotting was a valuable tool in this respect enabling visualization of those antigens recognized by patient immunoglobulins in serum or host fluids. The results from 4 burn patients indicated that several of the *P. aeruginosa* OMPs, notably the IRMPs, D E and H elicited an antibody response at an early phase of the infection. The early recognition of IRMPs was also noted in a rabbit model of disseminated peritonitis. The response to protein F appeared to develop at a later stage which may reflect destruction of certain epitopes of the porin protein following complete denaturation. Several workers have demonstrated the potential of this protein as a protective antigen (Gilleland *et al*, 1984; Matthews Greer *et al*, 1987). This study has identified additional proteins which may be of value in vaccine preparations.

Immunoblotting studies alone tell us nothing of the protective nature of the antibodies so formed although one can postulate, for example, that antibodies directed against receptors required for uptake of vital nutrients and cations such as iron may reduce the organisms ability to proliferate (Griffiths *et al*, 1983). Indeed antibodies to high molecular weight IRMPs in *E. coli* were recently shown to confer passive protection of turkeys challenged with the same strain (Bolin and Jensen, 1987). The true value of *P. aeruginosa* IRMPs as vaccine candidates can only be evaluated by purifying the proteins for active immunization protocols or raising antisera for passive therapy. Their protective capacity must then be tested against challenge with heterologous serotypes of the organism in a realistic animal model of infection. Preliminary studies were carried out to define optimum methods for extraction of the proteins from the OM and subsequent separation from other OMPs and contaminating LPS. The techniques described were successful at an analytical level although their potential for large scale production remains to be seen. Other techniques may show

even more promise and the molecular genetic approach in particular may overcome many problems regarding bulk production of iron-limited bacteria. Monoclonal antibodies to the proteins offer exciting prospects both for passive immunotherapy and for purification purposes.

Immunotherapy of infections due to *P. aeruginosa* is an attractive proposition considering the marked resistance of the organism to many antimicrobial agents. However, it may not be the answer in all types of infection. For example, considerable doubts exist as to the benefits of vaccination in patients with cystic fibrosis after their lungs become colonized with *P. aeruginosa*. These patients already have high titres of antibodies to bacterial antigens and vaccination may further encourage formation of destructive immune complexes. It seems that antibodies are most effective at opsonizing free forms of the bacteria such as occur in acute burn wound infection and septicaemia, but cannot assist in the eradication of sessile bacteria enclosed in the extensive glycocalyx-enclosed microcolonies seen in the CF lung. The biofilm mode of growth of *P. aeruginosa* is also commonly observed following colonization of implanted prostheses, including peritoneal dialysis catheters. The bacteria embedded in the biofilm display a greater resistance to antibiotics and host defences than corresponding planktonic cells. In an animal model of biomaterial-associated infection it became apparent that, in addition to the latter factor, bacteria initially succeeded in multiplying within the biofilm and attaining a high population of cells without evoking a strong immune response. This was in contrast to the free forms of bacteria in a disseminated peritonitis model and may help to explain the persistence of such infections. A nidus of bacteria is thus present which may at any time shed mobile cells to cause recurrent episodes of widespread peritonitis. In such patients it may be of more benefit to discover methods to prevent initial colonization of the prosthetic devices by incorporation of

antibiotics into the biomaterials during polymerization (Troskin *et al*, 1985). Alternatively, elucidation of methods to enhance the activity of phagocytic cells such that they can more effectively destroy the bacteria within biofilms may prove a greater success.

9. References

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