CHAPTER 4

Outer Membrane Permeability of *Pseudomonas aeruginosa*

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I. Introduction

It has been common knowledge that most strains of *Pseudomonas aeruginosa* are resistant to a number of commonly employed antibiotics, and very often also to a number of other agents including surfactants (Brown, 1975; Bryan, 1979). Until very recently we did not possess much solid data on the basis of this resistance. However, several perceptive scientists noted that a similar resistance, although quantitatively much less impressive, was a general property of most gram-negative bacteria, and they tried to understand the resistance of *P. aeruginosa* in the general context of what was known about the structure and properties of the surface layer uniquely present in gram-negative bacteria, i.e., the outer membrane (Brown, 1975; Bryan, 1979). One might infer that the outer membrane could frequently act as an effective permeability barrier because disruption of this membrane with detergents or EDTA, or introduction of mutations affecting components of this membrane, very often led to a strong increase in the sensitivity of bacteria to various agents (Leive, 1974; Richmond and Curtis, 1974; Brown, 1975; Nikaido, 1976). It was thus hypothesized that the outer membrane of *P. aeruginosa* acts as an unusually effective permeability barrier (Brown, 1975; Bryan, 1979). Although few direct results were available, we can cite, with hindsight, the finding that *P. aeruginosa* cells containing chloramphenicol acetyl transferase (presumably on the external surface of the cytoplasmic membrane) do not release modified chloramphenicol molecules into the medium, in contrast to *Escherichia coli* cells, which convert all chloramphenicol molecules in the medium into the acetylated form (Mitsuhashi *et al.*, 1975). This is one of the few early experiments which showed definitively that *P. aeruginosa* outer membrane had a much lower permeability than the *E. coli* outer membrane, and therefore the enzyme in the periplasm of the former organism reacted only with a few molecules of the drug that trickled through the outer membrane barrier.

The past several years witnessed a great expansion of our knowledge on the structure and function of the *P. aeruginosa* outer membrane, and now we understand, at least to a certain extent, the more effective barrier properties of this membrane at the molecular level. We shall try, in the following sections, to describe the architecture and components of the *P. aeruginosa* outer membrane in the larger context of our knowledge on the outer membrane of other gram-negative bacteria, especially that of enteric bacteria.

II. Isolation and Composition of the Outer Membrane

A. Isolation of Outer Membrane

Outer membranes of most gram-negative bacteria can be separated from the inner, cytoplasmic membrane by equilibrium sucrose density gradient centrifugation. Several methods have been proposed for the isolation of outer membrane from *P. aeruginosa* (Stinnett and Eagon, 1973; Booth and Curtis, 1977; Mizuno and Kageyama, 1978). These methods, however, were not completely satisfactory because they involve the use of EDTA, which removes a large fraction of the lipopolysaccharide and possibly some other components from the outer membrane (Rogers *et al.*, 1969; Roberts *et al.*, 1970). We devised a method that avoids the use of this chelating agent (Hancock and Nikaido, 1978). In this method, the cells are plasmolyzed in 20% sucrose, and are broken by passage through a French press. The resulting crude membrane fraction is then separated in a discontinuous density gradient of sucrose in Tris–HCl buffer, in the total absence of EDTA. This method is very reliable, and was successfully used by us as well as several other laboratories for the isolation of outer membranes from several unrelated strains of *P. aeruginosa*. As in other gram-negative bacteria (Nikaido and Vaara, 1985), the outer membrane of *P. aeruginosa* contains three major constituents, phospholipids, lipopolysaccharide, and proteins. The earlier literature on the outer membrane of this organism has been reviewed by Meadow (1975).

B. Phospholipids

The chloroform–methanol-extractable lipids of the outer membrane of *P. aeruginosa* mostly consist of phospholipids (Meadow, 1975). The predominant species is phosphatidylethanolamine, and this is supplemented with smaller amounts of acidic phospholipids, phosphatidylglycerol, and diphosphatidylglycerol (cardiolipin), as are found in many other gram-negative bacteria, as well as an unknown phospholipid. In terms of fatty acid composition, the major unsaturated fatty acid is *cis*-vaccenic acid (C18 : 1) rather than the palmitoleic acid (C16 : 1) (and its derivative C17 cyclopropane fatty acid) that is most abundant in other gram-negative bacteria grown at 37°C (Hancock and Meadow, 1969). This may affect the viscosity, and therefore the permeability, of the hydrocarbon interior of the *P. aeruginosa* outer membrane, but this point does not seem to have been investigated.

C. Lipopolysaccharide

As in *Enterobacteriaceae*, the lipopolysaccharide molecule consists of the hydrophobic lipid A portion, the hydrophilic core oligosaccharide, and the strain- or serotype-specific O-chain polysaccharide that forms the most peripheral portion of the molecule and protrudes into the external medium. *Pseudomonas aeruginosa* lipopolysaccharides can be extracted and purified by methods developed for enterobacterial lipopolysaccharides (Galanos *et al.*, 1969; Westphal and Jann, 1965). However, as with enterobacteriaceal strains, R-type or

core-only lipopolysaccharides and those carrying long O side chains usually coexist in the same strain, and the quantitative recovery of both of these types becomes difficult. For such situations, Darveau and Hancock (1983) devised a method of isolation that involves the sodium dodecyl sulfate (SDS) solubilization of lipopolysaccharide and removal of envelope proteins by protease digestion. Using this purification procedure, it has been estimated that only between 10 and 25% of lipopolysaccharide molecules from wild-type *P. aeruginosa* strains are capped with O side chains (Hancock *et al.*, 1983a; A. Kropinski, personal communication).

It has been shown that the O-polysaccharide portion of *P. aeruginosa* lipopolysaccharide often contains such sugars as glucose, rhamnose, glucosamine, fucosamine, and quinovosamine. In addition, other studies revealed the presence, in many serotypes, of such rare sugars as aminohexuronic acids (Koval and Meadow, 1975; Wilkinson and Welbourn, 1975) and 2,4-diamino-2,4,6-dideoxyhexose (Wilkinson, 1977). In recent years, further advances have been made in our understanding of the structure of the O-chain portion of this molecule by the work of Dmitriev, Kochetkov, and their co-workers (Dmitriev *et al.*, 1980, 1982; Knirel *et al.*, 1982a,b, 1983). As shown in Table I, these studies showed that chains of *P. aeruginosa* lipopolysaccharide often contain additional unusual sugars such as 2-imidazolinomannuronic acid or 2,3-diacetamido-2,3-dideoxyhexuronic acid. Even more important, these studies substantiated the earlier conclusions of Meadow and colleagues (Chester *et al.*, 1973; Koval and Meadow, 1975) in showing that the serological classification of *P. aeruginosa*,

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2a,c		4)L-GalNAcUA(α1–3)D-QuiNAc(α1–3)L-Rha(α1–3)L-Rha(α1-
3a,b	16	4)D-ManImUA(β1–4)D-Man(NAc)$_2$UA(β1–3)D-FucNAc(β1-
3(a),c	2	4)D-ManImUA(β1–4)L-Gul(NAc)$_2$UA(α1–3)D-FucNA(β1-
3a,d	5	4)D-ManImUA(β1–4)D-Man(NAc)$_2$UA(β1–3)D-FucNAc(α1-
3a,d,e		4)D-ManImUA(β1–4)L-Gul(NAc)$_2$UA(α1–3)D-FucNAc(α1-
3(a),d,f		4)D-ManImUA(β1–?),Hex(NAc)$_2$UA,D-FucNAc
6	1	4)D-GalNAc(α1–4)D-Glc(NAc)$_2$UA(β1–3)D-FucNAc(α1–3)D-QuiNAc(α1-
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especially that of Lanyi (1966), correlates well with the structural differences in the O-chain portion of the lipopolysaccharide. Strain PAO1, which has been used most frequently in genetic and outer membrane studies, was recently shown (Kuzio and Kropinski, 1983) to have an O-antigen composition identical to the Lanyi 3a,d type.

Our knowledge of the structure of the core portion of the molecule is somewhat more limited. When the lipid A portion is split off from the polysaccharide part by mild acid hydrolysis, gel filtration separates the polysaccharides into two fractions: a high molecular weight fraction showing varying composition depending on the serotype of the strain, and therefore presumably corresponding to the core oligosaccharides capped by long specific O-polysaccharide chains, and a low molecular weight fraction containing always similar sugars and therefore presumably corresponding to the "uncapped" core oligosaccharides (Chester *et al.*, 1973; Drewry, *et al.*, 1975). The sugars found in the low molecular weight fraction are 3-deoxy-D-mannooctulosonic acid (or 2-keto-3-deoxyoctonic acid, KDO). an aldoheptose (presumably L-glycero-D-mannoheptose found in the lipopolysaccharide of Enterobacteriaceae), glucose, rhamnose, and galactosamine. In addition, this fraction contains phosphate and alanine. The suggestion that this fraction corresponds to the core oligosaccharide was also supported by the finding that these same components were constantly present in the lipopolysaccharide of any serotype, whereas such sugars as quinovosamine and fucosamine, were present only in the lipopolysaccharides of a few serotypes (Chester *et al.*, 1973; Wilkinson, 1983). Quantitation of various components in this fraction, performed in various laboratories by using samples derived from different strains and serotypes, demonstrated remarkable agreement in showing that, per one residue of galactosamine, there are 2–3 heptose, 1–2 rhamnose, 3–4 glucose, 1–2 alanine, 6–7 phosphate, and 1–3 KDO residues (Chester *et al.*, 1973; Wilkinson and Galbraith, 1975; Meadow and Wells, 1978; Kropinski *et al.*, 1979).

Further analysis of core structure was made possible by the isolation of different types of mutants defective in core synthesis, through the use of pyocins and bacteriophages specific for various types of core structures. In this manner, Meadow and Wells (1978) as well as Kropinski *et al.* (1979) came to the conclusion that glucose and rhamnose residues must occupy relatively distal positions in the core (see Fig. 1). The former workers could isolate a mutant totally lacking rhamnose but otherwise unaltered in the content of other core sugars, and thus concluded that rhamnose occupied only the outermost position(s). More recently, Rowe and Meadow (1983) proposed a detailed structure of the core region of strain PAC1, based on the methylation and NMR analysis of oligosaccharides obtained from various mutants (Fig. 1). This is the most precise description of the core structure in *P. aeruginosa* to date. However, we note that this structure does not fit with the isolation of glucosylrhamnosylglucose trisac-

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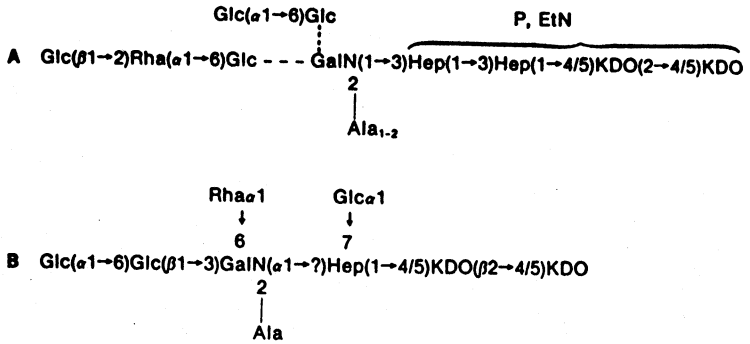

FIG. 1. Partial structures proposed for the core oligosaccharides of lipopolysaccharide from *Pseudomonas aeruginosa* strains NCTC 1999 (A) (Drewry *et al.*, 1975) and PAC1R (B) (Rowe and Meadow, 1983). (Reproduced from Wilkinson, 1983, with permission.) Abbreviations: KDO, 2-keto-3-deoxyoctulosonate; Hep, heptose; GalN, galactosamine; Rha, rhamnose; Ala, alanine; Glc, glucose; P, phosphate; EtN, ethanolamine.

charide from partial acid hydrolyzate of lipopolysaccharide from strain NCTC 1999 (Drewry *et al.*, 1975). Possibly the core portion from different strains is similar in composition, but the detailed structure, i.e., the manner in which the component sugars are linked together, is not identical (as suggested in Wilkinson, 1983); such a situation was indeed found previously in the core oligosaccharides of enteric bacteria (Jansson *et al.*, 1981). Rough core heterogeneity in *P. aeruginosa* has also been indicated by the isolation of two rough-core-specific monoclonal antibodies which react with some but not all of the *P. aeruginosa* strains tested (Hancock *et al.*, 1983a).

The phosphate content of the isolated core polysaccharide seems to be much higher than that of *Enterobacteriaceae*, such as *Salmonella minnesota*, where the presence of only one or two phosphate residues per chain, containing two or three heptose residues, has been reported (Droge *et al.*, 1968). The difference may in fact be much larger, because acetic acid hydrolysis for the removal of lipid A appears to release a very large amount of phosphate and phosphorylated compounds, including ethanolamine triphosphate (Drewry *et al.*, 1972). Analysis of whole lipopolysaccharide suggests that there are 11–16 phosphate residues per chain of core oligosaccharide, which contains one galactosamine residue (Drewry *et al.*, 1975; Kropinski *et al.*, 1979). Although we are not aware of studies in which enterobacterial lipopolysaccharides have been studied in parallel in the same laboratory, these numbers seem very large indeed in comparison with *S. minnesota* and *E. coli* lipopolysaccharides, for which values of 4–5 (Droge *et al.*, 1968) and 6–7 (Prehm *et al.*, 1976) have been reported, respectively. It therefore seems important to determine the location and mode of linkage of these phosphate units in *P. aeruginosa* lipopolysaccharide. Preliminary data with *P.*

aeruginosa PAO1 LPS extracted by the method of Darveau and Hancock (1983) has suggested that 6.5% of the phosphate is present as triphosphate, 73.2% as pyrophosphate monoester, 1.5% as pyrophosphate diester, and 23.1% as monophosphate, including about 10% associated with lipid A (J. Redmond and R. E. W. Hancock, unpublished observations). The negative charges of some of these phosphate units are undoubtedly neutralized by the positive charges of ethanolamine and possibly of alanine. However, the presence of these many negatively charged groups may certainly contribute to the exceptional sensitivity of *P. aeruginosa* to chelating agents such as EDTA (Gray and Wilkinson, 1965), because removal of divalent cations acting as bridges between neighboring lipopolysaccharide molecules would create strong electrostatic repulsion, which would result in the destabilization of the outer membrane and in the release of the lipopolysaccharide into the medium (Peterson *et al.*, 1985).

The lipid A of *Enterobacteriaceae* consists of a 4-phosphoglucosaminyl-(1→6)-glucosamine 1-phosphate backbone, to which several fatty acid chains are attached via ester and amide linkages. The detailed structure of this lipid A has recently been established through a combination of purification by high-pressure liquid chromatography, fast atomic bombardment mass spectrometry, and nuclear magnetic resonance, as well as by organic synthesis (Imoto *et al.*, 1983; Qureshi *et al.*, 1983; Strain *et al.*, 1983; Takahama *et al.*, 1983). In this structure (Fig. 2), the fatty acid residues attached directly to the disaccharide backbone are all 3-OH-tetradecanoic acids, a component that occurs in the lipopolysaccharide of most gram-negative bacteria. Interestingly, in *P. aeruginosa* there is little of this fatty acid, which is replaced mainly by 2-OH- and 3-OH-dodecanoic acids and some 3-OH-decanoic acid (Meadow, 1975). Since there is evidence that the hydroxyl groups of either 3-OH-dodecanoic acids

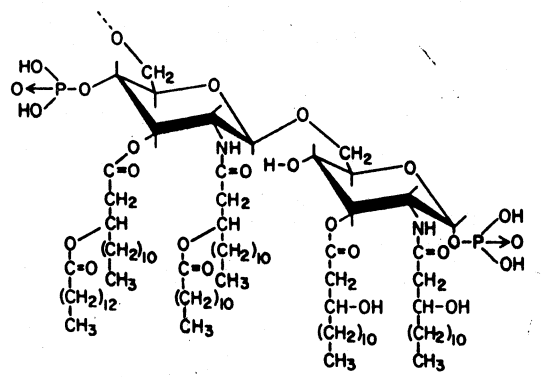

FIG. 2. Structure of lipid A from *Salmonella typhimurium* lipopolysaccharide. (Reproduced from Takayama *et al.*, 1985, with permission.) It is suggested in the text that *Pseudomonas aeruginosa* lipid A is similar, although the fatty acyl chains attached to the diglucosamine are shorter by 2–4 carbons.

(Drewry *et al.*, 1973) or 3-OH-decanoic acids (Pier *et al.*, 1981) are substituted by other fatty acids, it is most likely that the basic structure of the *P. aeruginosa* lipid A is similar to that of the enterobacterial lipid A shown in Fig. 2, except that the chains of the hydroxy fatty acids are shorter. Evidence favoring this similarity includes antigenic cross-reactivity at the monoclonal level (Mutharia *et al.*, 1984) and nearly identical infrared spectra (Horton *et al.*, 1977).

If we consider that the shortening of fatty acid chains by two carbons lowers the melting points of phospholipids by 20°–30°C, it is expected that the hydrophobic portion of the *P. aeruginosa* lipopolysaccharide would show a higher fluidity than that of the enterobacterial lipid A. Another peculiarity of lipid A from *P. aeruginosa* and several other *Pseudomonas* species is the presence of a 2-hydroxy fatty acid, 2-OH-dodecanoic acid (Meadow, 1975). This fatty acid appears to occur as a substituent of the 3-hydroxyl groups of 3-OH-dodecanoic acid residues (Drewry *et al.*, 1973), and its presence might further hinder the tight packing of the fatty acid chains of lipid A in this organism. We mentioned earlier that the core portion of the lipopolysaccharide of this organism is likely to show a strong lateral interaction because of the presence of a large number of phosphate groups. The combination of stronger interaction in the hydrophilic region with the more fluid hydrophobic interior is certain to modify the barrier properties of the lipopolysaccharide monolayer (see below) in significant ways, but at present our data on the permeability or barrier properties of the lipid domains of the *P. aeruginosa* outer membrane is incomplete.

In enteric bacteria, covalent labeling studies with reagents impermeable through the outer membrane (Kamio and Nikaido, 1976) as well as consideration of the numbers of lipopolysaccharide and phospholipid fatty acid chains present (Smit *et al.*, 1975) suggested strongly that the outer half of the lipid bilayer of the outer membrane consists almost entirely of lipopolysacchrides. Presumably the lateral interactions between the neighboring lipopolysaccharide molecules are very strong because of the bridging effect of divalent cations in the aqueous domain and the tight and regular packing of saturated fatty acids of the lipid A; this strong interaction would explain the unusually low permeability of the enteric outer membrane to hydrophobic compounds, such as hydrophobic antibiotics, dyes, and detergents including bile salts (Nikaido, 1976; Nikaido and Vaara, 1985). Unfortunately, we have few data on the density of lipopolysaccharide and phospholipid molecules in the *P. aeruginosa* outer membrane, nor has the permeability of *P. aeruginosa* outer membrane toward hydrophobic compounds been investigated in a systematic manner. However, in view of the high resistance of *P. aeruginosa* to bile salts, dyes, and hydrophobic antibiotics (see below), it seems most likely that the outer membrane of this organism is also organized in a manner similar to that of enteric organisms, i.e., with an asymmetric lipid bilayer containing essentially only lipopolysaccharide molecules in its outer leaflet.

D. PROTEINS

As in enteric bacteria, the outer membrane of *P. aeruginosa* seems to contain a large number of proteins, yet its protein pattern is dominated by several "major" proteins (Fig. 3). Various schemes for the nomenclature of these proteins have been reconciled by Hancock and Carey (1979) into one based on that of Mizuno and Kageyama (1978), with modifications that resulted from the improved reso-

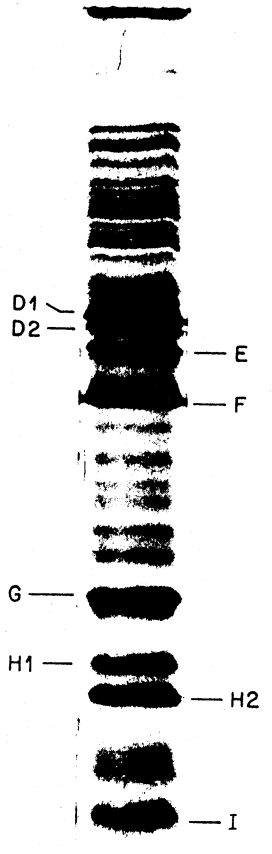

FIG. 3. SDS–polyacrylamide gel electrophoretogram of the major outer membrane proteins of *Pseudomonas aeruginosa* after growth on BM2 glucose. See Hancock and Carey (1979) for the effect of growth conditions and solubilization temperatures on the observed pattern of major outer membrane proteins. Although protein I is the outer membrane protein present in the highest copy number, it is stained poorly in this gel due to masking by the rough core of lipopolysaccharide (Angus *et al.*, 1982).

lution of their gel system (Fig. 3). We comment on some of these proteins briefly.

1. Protein F (Porin)

This protein was shown to be the major protein responsible for the formation of nonspecific, water-filled pores across the outer membrane through reconstitution (Hancock *et al.*, 1979) and mutant (Nicas and Hancock, 1983a) studies. It will be described in detail below. However, we note here that, unlike the enterobacterial porins that remain trimeric in the presence of SDS at moderate (below about 70°C) temperatures (Nakae *et al.*, 1979), protein F migrates as monomers when analyzed by SDS–polyacrylamide gel electrophoresis after extraction with SDS at room temperature (Mizuno and Kageyama, 1978; Hancock *et al.*, 1979).

2. Protein I (Lipoprotein)

Pseudomonas aeruginosa outer membrane contains a small protein which is similar to the murein lipoprotein of *E. coli* in mobility in SDS–polyacrylamide electrophoresis, presence of covalently bound glycerol and fatty acid residues, predominantly α-helical secondary structure, and amino acid composition (Mizuno and Kageyama, 1979). Like *E. coli* murein lipoprotein, protein I lacks any proline, cysteine, phenylalanine, and tryptophan residues. However, protein I contains one residue each of histidine and glycine, which are absent in murein lipoprotein (Mizuno and Kageyama, 1979). Mizuno and Kageyama (1979) reported that free protein I could be completely extracted from the cell envelope with SDS at 60°C, a result suggesting that protein I interacts with the peptidoglycan layer in a noncovalent manner. Since SDS was found to release much of the protein I at 35°C, but not at 23°C, from Triton-extracted protein–peptidoglycan complexes (Hancock *et al.*, 1981a), it is possible that this noncovalent interaction is a very weak one. Some of the protein I molecules were reported to occur in a form covalently linked to peptidoglycan, but no quantitative information was given (Mizuno and Kageyama, 1979). If the covalently bound form indeed occurs, it should represent an exceedingly small fraction of the population, as we have consistently failed to show the presence of this form in *P. aeruginosa* strain PAO1 (Chen *et al.*, 1980; Hancock *et al.*, 1981a). In *E. coli*, the murein lipoprotein is assumed to play a structural role in the stabilization of outer membrane structure, and possibly protein I has a similar function in *P. aeruginosa*.

3. Protein H2

Mizuno (1979) found that the protein migrating with an apparent molecular weight of 21,000 was a lipoprotein, with covalently linked fatty acid and glycerol residues. He also found a similar lipoprotein in *E. coli* as well as several species of enteric bacteria. The *P. aeruginosa* protein remains noncovalently associated with the peptidoglycan layer after extraction of other proteins with SDS at 30°C (Mizuno, 1979), a finding confirmed later by using a different approach (Hancock *et al.*, 1981a). This band was resolved into two proteins, H1 and H2, by Hancock and Carey (1979), and the lipoprotein was found to correspond to protein H2. The function of this protein is unknown, but it is strongly antigenically conserved in related *Pseudomonas* sp. (Mutharia *et al.*, 1985b).

4. Proteins Induced under Special Conditions

Protein P, which apparently produces an anion-specific channel, has an apparent molecular weight of 47,000 and is produced under phosphate starvation conditions (Hancock *et al.*, 1982). Protein D1, with an apparent molecular weight of 46,000, was found to be produced in large amounts only when the cells were grown in media containing glucose (Mizuno and Kageyama, 1978). It is thought to function as an efficient channel for glucose (Hancock and Carey, 1980). These two channel-forming proteins are discussed in Section IV. Protein H1 was found to be increased greatly in polymyxin- and EDTA-resistant mutants, and is thought to substitute for divalent cations in stabilizing the structure of the outer membrane (Nicas and Hancock, 1980). This is not a peptidoglycan-associated protein (Hancock *et al.*, 1981a). The function of this protein is discussed in Section VI,C. When *P. aeruginosa* is grown in iron-deficient medium, two outer membrane proteins of 75,000 and 80,000 daltons are induced, as is the iron transport siderophore pyoverdine (Meyer *et al.*, 1979). The authors proposed, by analogy to *E. coli* data (Hancock *et al.*, 1976), that these outer membrane proteins are involved in the transport of iron–siderophore complexes.

5. Outer Membrane-Associated Enzymes

Ohkawa *et al.* (1979) demonstrated the presence of a long-chain acyl esterase as a minor component of the outer membrane of *P. aeruginosa*. The enzyme, a 55,000-dalton protein, cleaved long-chain acyl-CoA thioesters as well as some kinds of oxyesters, including Tween 80, but did not hydrolyze triglycerides or phospholipids. Mutants lacking the esterase activity were unable to grow on Tween 80 as a sole carbon source, suggesting a possible role of the esterase in the utilization of acyl esters as a carbon source.

Kageyama and colleagues (Mizuno and Kageyama, 1978; Ohkawa *et al.*, 1979) also found a distinct detergent-resistant phospholipase A activity in their outer membrane preparations. Although this was not characterized in detail, it seemed to be analogous to the previously described outer membrane-associated phospholipase activity of *E. coli* (Nishijima *et al.*, 1977).

6. "Heat-Modifiable" Proteins

In the outer membranes of enteric bacteria, there is usually a major outer membrane protein which characteristically shows reduced mobility after heating in SDS at 100°C. This behavior, which was called "heat modifiability" in the earlier literature, presumably reflects the fact that the protein does not unfold completely in the presence of SDS at low temperatures, perhaps because much of the surface of the protein is hydrophobic and SDS binding to these domains does not distort the conformation, and/or because the protein has unusually stable secondary and tertiary structures. In *E. coli*, the only major protein showing such behavior is OmpA, which is thought to play a structural role in the outer membrane (Nikaido and Vaara, 1985). Proteins which show immunological cross-reactions with OmpA and therefore are homologous to it have been demonstrated in various other species of Enterobacteriaceae (Hofstra and Dankert, 1980). In contrast, *P. aeruginosa* outer membrane contains many proteins showing the heat-modifiable phenotype (Mizuno and Kageyama, 1978; Hancock and Carey, 1979). In addition to porin (protein F), D1, and H1, which have already been mentioned, proteins D2 and G show this behavior, with their apparent molecular weights shifting from 35,500 to 45,500 and from 19,000 to 25,000, respectively, on heating in SDS (Hancock and Carey, 1979). However, none of these proteins showed immunological cross-reaction with the *E. coli* OmpA protein (Hofstra and Dankert, 1980).

III. Permeation of Hydrophilic Compounds through the Outer Membrane: The Role of Protein F

A. Protein F: The Major Porin Protein

In *E. coli* and *Salmonella typhimurium*, Nakae (1976a,b) used reconstitution, with phospholipids and lipopolysaccharides, of purified outer membrane proteins as well as outer membrane fragments to show that some major outer membrane proteins form water-filled, nonspecific, transmembrane diffusion channels. He named these proteins porins. The same approach was utilized with *P. aeruginosa*. Its outer membrane was isolated in the absence of EDTA (Hancock and Nikaido, 1978), and small fragments of this membrane were reconstituted with lipopolysaccharides and phospholipids in the presence of [^{14}C]sucrose, which was thought to penetrate through the porin channel, and [^{3}H]inulin, which was expected to be impermeable from the results with enterobacterial porins. The resulting proteoliposomes, however, did not show significant differential retention of the inulin when examined after extensive washing. We hypothesized that this could be due to the unusually large size of the channel, which could have

allowed the efflux of inulin, and therefore we used instead [^{3}H]dextran of large size. The proteoliposomes reconstituted in this manner lost most of the internal [^{14}C]sucrose during the washing step on a Millipore filter, due to efflux through the porin channels, but retained much of the [^{3}H]dextran inside the vesicles. This demonstrated that *P. aeruginosa* contained a component capable of producing channels that allowed the diffusion of hydrophilic solutes (Hancock and Nikaido, 1978).

In the reconstitution experiments mentioned above, the component contributed by the outer membrane was protein(s), because reconstitution from lipopolysaccharide and phospholipid alone did not produce vesicles that allowed the efflux of sucrose. The identification of the protein, or porin, was carried out by purifying the outer membrane proteins by ion-exchange chromatography on DEAE–cellulose in the presence of Triton X-100, and by reconstituting proteoliposomes with these partially purified protein fractions (Hancock *et al.*, 1979). The results showed clearly that only the protein F had pore-forming activity.

Demonstration of the pore-forming activity of a protein in a reconstitution experiment does not necessarily prove that it is indeed the major porin functioning in the intact cell. This becomes an especially serious question with the *P. aeruginosa* porin, because its "specific activity" in creating permeability in liposome membranes is unusually low, as discussed in Section III,C. However, there are several pieces of evidence that identify protein F as the major porin of *P. aeruginosa:* (1) the size of the channel, as determined by the exclusion of saccharides of various sizes, is similar for intact outer membranes (Decad and Nikaido, 1976), proteoliposome membranes reconstituted from unfractionated outer membrane fragments (Hancock and Nikaido, 1978), and proteoliposome membranes reconstituted from purified porin (Hancock *et al.*, 1979); (2) the low permeability of the outer membrane in intact cells is reflected by the low permeability of liposome and black lipid film membranes reconstituted from purified protein F, as described in Section III,C; and (3) when a protein F-deficient mutant was isolated, by screening SDS–polyacrylamide electrophoretograms of membrane proteins from 500 mutagenized clones of *P. aeruginosa*, it was found that the permeability of its outer membrane to a chromogenic cephalosporin, nitrocefin, was reduced by at least 80% in comparison with the wild type or protein F-producing revertants (Nicas and Hancock, 1983a). This is the most direct proof that the protein F is responsible for most of the nonspecific permeability properties of *P. aeruginosa* outer membrane.

B. Protein F and Large Channel Formation

Miller and Becker (1978) demonstrated that a methionine auxotroph of *P. aeruginosa* grew as well on pentamethionine as it did on the free amino acid

methionine. In unpublished experiments (R. V. Miller, personal communicaton) it was possible to obtain growth on hexa- and heptamethionine. In contrast, an *E. coli* methionine auxotroph grew on trimethionine and methionine-containing tetrapeptides but did not grow on pentamethionine (Becker and Naider, 1974) Since the peptidases which eventually cleave these methionine-containing peptides are either cytoplasmic (in the case of *E. coli*) or tightly bound to the cytoplasmic membrane (Haas *et al.*, 1981), the most likely explanation for these data is that the *P. aeruginosa* outer membrane has a larger exclusion limit than does the *E. coli* outer membrane, in that the former allowed relatively free passage of oligopeptides of sizes larger than the exclusion limit of *E. coli* porins. As described below, this interpretation is consistent with the majority of the published *in vitro* data on the respective porins of *E. coli* and *P. aeruginosa*.

Several different *in vitro* approaches have been used so far to estimate the size of the channel produced by protein F. All of them indicate that at least a portion of protein F channels are much larger than the channels produced by *E. coli* and *S. typhimurium* porins. In the first method, proteoliposomes containing ^{3}H-labeled saccharides of various sizes as well as [^{14}C]sucrose were reconstituted from *P. aeruginosa* phospholipids, *P. aeruginosa* lipopolysaccharide, and either fragments of outer membrane or purified protein F, as described earlier (Hancock and Nikaido, 1978; Hancock *et al.*, 1979). Because lipopolysaccharide molecules bear multiple negative charges (see Section II,B), membranes containing lipopolysaccharides show strong electrostatic repulsion against each other, and therefore this method tends to produce largely unilamellar vesicles (Nakae, 1976). These vesicles were then collected by filtration on Millipore filters and were washed repeatedly with a buffer. During this process, there was a nearly complete efflux of sucrose (M_r = 342) through the porin channel. In contrast to the *E. coli* or *S. typhimurium* porin channels, which are essentially impermeable to the tetrasaccharide stachyose (M_r = 666), inulin fractions with average molecular weights of 1700 and 2500 showed nearly complete efflux through the *P. aeruginosa* porin channel (Fig. 4). Although it was difficult to get a precise "exclusion size," partly because of the somewhat polydisperse nature of the polysaccharide samples used, we had to use a dextran of average molecular weight 10,000 in order to get nearly complete retention of the saccharide within vesicles, and the exclusion size can be estimated as several thousand daltons (see Fig. 4).

The radioisotope efflux assay described above has poor time resolution and does not give much information about the actual rates of diffusion of solutes through the channel. In order to get information of the latter type, the proteoliposome swelling assay was devised (Nikaido and Rosenberg, 1983). In this assay, multilayered proteoliposome vesicles are made from porin (or outer membrane fragments) and phospholipids in the presence of large solutes impermeable through the channel. When the vesicles are diluted into isotonic solutions of test

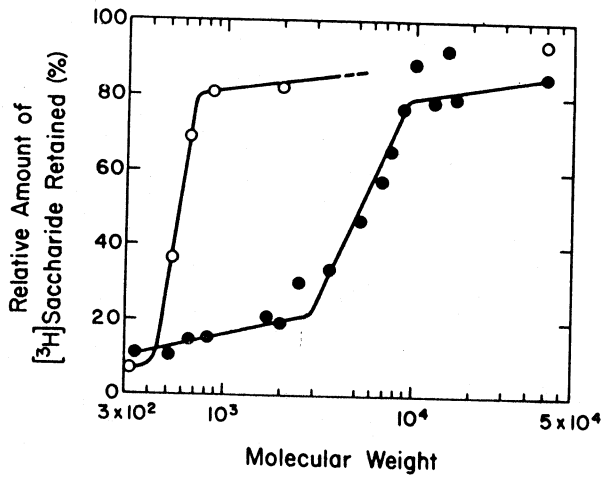

FIG. 4. Retention of oligosaccharides of various sizes in the intravesicular space after extensive washing proteoliposomes reconstituted with outer membrane fragments of *Pseudomonas aeruginosa* (solid circles) or purified *Escherichia coli* OmpF porin (open circles). (Reproduced from Hancock and Nikaido, 1978, with permission.) Purified protein F gave results similar to those obtained with outer membrane fragments (Hancock *et al.*, 1979).

solutes, the influx of test solutes through the porin channel produces an osmotic swelling of the vesicles, the rate of which can be determined from optical density tracing of the vesicle suspension. This rate, within certain limits, is proportional to the rate of solute penetration through the porin channel. With water-filled cylindrical channels, the permeation rates of solutes are predicted to be a function of their size, due to their collision with the rims of the pore and to the viscous drag exerted by the walls of the pore (Renkin, 1954). Indeed, the rate of swelling of protein F-containing vesicles was dependent on the size of the sugar that was present in the external medium (Yoshimura and Nikaido, 1982) (Fig. 5), and comparison of the slope of the dependence curve with those predicted on theoretical grounds for various diameters of the pore showed that the best match was obtained with that for a diameter of 2.0 nm. This is 1.72 times larger than the estimated diameter of the *E. coli* K12 OmpF pore, 1.16 nm (Nikaido *et al.*, 1983). Since the weight of globular molecules should increase in proportion to the cube of their diameter, the expected exclusion limit for the *P. aeruginosa* pores on the basis of these results would be $600 \times (2.0/1.16)3 = 3072$ daltons, if we assume that the limit for *E. coli* is 600 daltons (Decad and Nikaido, 1976). This value is within the range found with the radioisotope efflux assay described above.

The third technique used was reconstitution into black lipid films (Benz and Hancock, 1981). Lipid bilayers were made across a small aperture in the septum

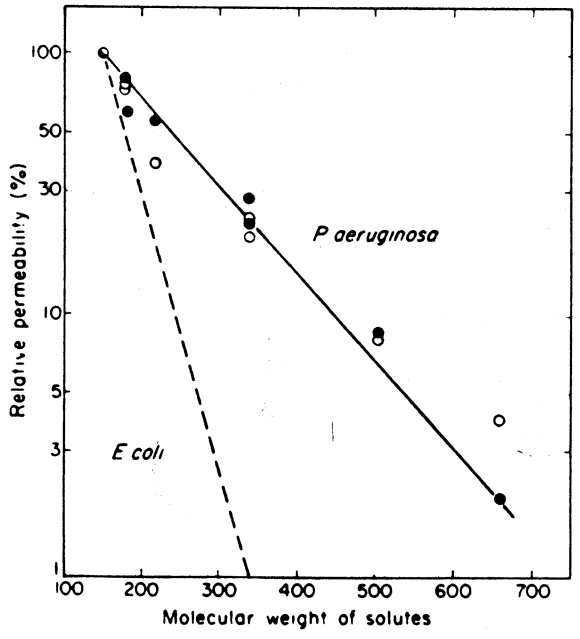

FIG. 5. Permeability of liposomes reconstituted from *Pseudomonas aeruginosa* purified protein F (solid circles) or a crude envelope fraction (open circles) as a function of the molecular weight of the sugar used to assess permeability. Typical results for *E. coli* OmpF protein are included for comparison (dashed line). (Reproduced from Yoshimura *et al.*, 1983, with permission.)

separating two aqueous compartments containing electrolyte solutions, and protein F, purified in the presence of Triton X-100, was added to the aqueous compartment. There was a gradual insertion of the protein into the bilayer, a phenomenon reflected by a stepwise increase in the electrical conductance across the bilayer (Fig. 6). The technique is a very powerful one because, due to the very high sensitivity of electrical measurements, one can observe the behavior of individual channels inserted into the bilayer. The single-channel conductance observed was 0.5 nS in 0.1 *M* NaCl (Fig. 6), or 5.6 nS in 1 *M* KCl. If one assumes that the KCl solution within the channel has the same conductivity as the bulk solution, the diameter of the channel is calculated to be 2.0 nm. With smaller channels, this method of calculation may be somewhat less reliable because the conductivity of salt solutions within narrow channels may not be identical with that of the bulk solution (Nikaido and Vaara, 1985). However, with channels as large as the protein F channel, this is not much of a problem, and we believe this estimate to be reliable. It is also satisfying that this estimate is very close to that obtained by the liposome swelling experiment. More recently, we have examined protein F purified by differential solubilization and electroelution from SDS polyacrylamide gel electrophoretograms (Woodruff *et al.*, 1986). Two sources of protein F were used: *P. aeruginosa* cells and *E. coli* cells containing the cloned protein F gene. By increasing the resolution of the lipid bilayer apparatus, we detected small channels (single channel conductance of 0.36 nS in 1 *M* KCl) as well as larger channels, as previously observed. These small channels were considered too small to allow permeation of even small substrates and thus would not have been observed in the liposome experiments described above (i.e., only the large channels would have been measured). We did not observe these channels in previous lipid bilayer studies (Benz and Hancock, 1981), since they would escape detection at the resolution of the instrumentation used to study the larger protein F channels. The smaller channel seemed to be more prevalent than the larger channel in the electroeluted preparations (Woodruff *et al.*, 1986).

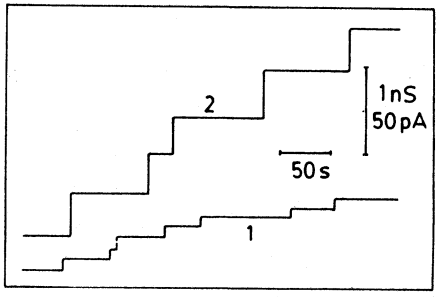

FIG. 6. Stepwise increase of the current across a lipid bilayer membrane in the presence of 0.1 ng/ml porin from *Escherichia coli* (trace 1) or 5 ng/ml porin F from *Pseudomonas aeruginosa* (trace 2), added to the aqueous phase containing 0.1 *M* NaCl; temperature, 25°C. The membranes were formed from egg phosphatidylcholine/*n*-decane and the applied voltage was 50 mV. Note the larger conductance steps (indicating larger channels) and poorer activity (50-fold more protein F was required to obtain a similar reconstitution rate) of protein F. A nanoSiemen (nS) is a unit of conductance equal to 10^{-9} Ohm^{-1}; pA, picoampere. [From R. Benz, R. E. W. Hancock, and T. Nakae, "Transport in Biomembranes: Model Systems and Reconstitution" (R. Anatolini, A. Gliozzi, and A. Gorio, eds.), copyright 1982 Raven Press, New York.]

Caulcott *et al.* (1984) measured the efflux of radiolabeled solutes from preequilibrated, plasmolyzed cells of *P. aeruginosa*. Because the kinetics of efflux with *P. aeruginosa* were similar to those obtained with *E. coli*, these authors concluded that the pores of *P. aeruginosa* were of the same size as those of *E. coli*, in contrast to the results so far described. We do not believe that their data warrant this conclusion. From the known permeability coefficients of the outer membrane, one can calculate the time needed for half-equilibration of solutes across the outer membrane (Nikaido *et al.*, 1983). This time is very short, at least for the outer membrane of *E. coli*, and usually on the order of a second or less for most solutes. Thus, at least for *E. coli* and probably also for *P. aeruginosa*, the kinetics of efflux of radiolabeled solutes that the authors measured in the time

range 15–30 min are unlikely to have anything to do with the diffusion across the outer membrane. Possibly they represent efflux from the cytoplasm of cells with damaged cytoplasmic membranes or dissociation of cell-bound radiolabeled solutes.

Lipid bilayer experiments allow determination of the ion preference of porin channels. Such studies (Benz and Hancock, 1981) showed that the large *P. aeruginosa* porin channel has a slight cation preference, with the cation permeability generally 2- to 2.7-fold higher than the anion permeability. This can be contrasted with the stronger cation selectivity of the narrower *E. coli* channels, especially that of the OmpC channel, which can show cation permeability up to 30 times higher than that for anions (Benz *et al.*, 1985).

The smaller degree of cation selectivity of the *P. aeruginosa* porin channel seems to be due at least in part to the larger size of the channel. Because of its size, the solute molecule is less likely to be influenced by the properties of the channel. This difference can become very pronounced when we are dealing with larger compounds that barely go through the *E. coli* channel. As an example, we show in Table II the rates of diffusion of a few cephalosporins through the *E. coli* OmpF porin channel, as well as *P. aeruginosa* outer membrane determined in intact cells. Although in *E. coli* the penetration rates are very strongly influenced by the properties of the solutes—for example, the net negative charge of cephacetrile produces a striking reduction in permeation rate in comparison with zwitterionic cephaloridine—the differences between these compounds are very small in the case of *P. aeruginosa* porin channel.

Another property of the porin channel which we should consider is that of

TABLE II
RATES OF PENETRATION OF SOME CEPHALOSPORINS ACROSS THE OUTER MEMBRANES OF *Pseudomonas aeruginosa* AND *Escherichia coli*[a]

	Permeability coefficients (cm/sec) for the outer membranes	
Cephalosporin	*P. aeruginosa*	*E. coli*[b]
Cephaloridine	12	5260
Cephacetrile	10	800
Nitrocefin	8	220

[a] The data are from Yoshimura and Nikaido (1982), Nikaido *et al.* (1983), and J. Hellman and H. Nikaido (unpublished observations). Similar data for nitrocefin were obtained by Nicas and Hancock (1983a).

[b] Containing only the OmpF porin.

gating. Schindler and Rosenbusch (1978, 1981) incorporated *E. coli* OmpF porin first into monolayers at the air–water interphase and then into planar bilayers. When introduced in this manner, the channel apparently closed when the potential across the membrane exceeded certain threshold values. It was proposed that this phenomenon of gating has some physiological function in the regulation of outer membrane permeability (Schindler and Rosenbusch, 1981). However, when the porins of *P. aeruginosa* (Benz and Hancock, 1981) or *E. coli* (Benz *et al.*, 1978) were introduced into bilayers from the aqueous phase, there was no indication of this gating phenomenon. Furthermore, in intact cells of *E. coli*, potentials up to 120 mV were shown not to influence the permeability of the outer membrane to a cephalosporin (J. Hellman and H. Nikaido, unpublished observations). We therefore believe that the observed gating is likely to be an artifact of the method of incorporation of the porin into the bilayers (Benz *et al.*, 1982).

C. Protein F and Low Outer Membrane Permeability

As described above, the diameter of a portion of the protein F channels is quite large. Also, the protein is one of the most abundant proteins in the outer membrane (see Fig. 2), corresponding to 15% of the total outer membrane protein under a set of typical growth conditions (Benz and Hancock, 1981). Thus, if the proportion of large channels was high, one would expect the permeability of the *P. aeruginosa* outer membrane to hydrophilic molecules to be quite high, in comparison with the enteric bacteria that produce porins with much narrower channels. However, it is well known that *P. aeruginosa* shows high resistance to a number of antibiotics and other inhibitors, and it has been assumed that this resistance is due to the low permeability of the outer membrane (see Section I). To explain this apparent contradiction, Benz and Hancock (1981) proposed that a large fraction of the protein F consists of closed channels. An alternative explanation might be that the pores are large but have high resistance to penetration, perhaps because of the tortuous path the channel follows. This explanation, however, was disproved by the observation of the high single-channel conductivity induced by the F protein in the black bilayer system (Benz and Hancock, 1981). As discussed above, there is now evidence suggesting that a large fraction of protein F channels are small rather than closed (Woodruff *et al.*, 1986). However, such small channels should be essentially impermeable to large hydrophilic compounds like antibiotics.

The first concrete experimental evidence concerning the presumed low permeability of the *P. aeruginosa* outer membrane was obtained by Angus *et al.* (1982) as well as by Yoshimura and Nikaido (1982). In the former study, the permeability of *P. aeruginosa* outer membrane to a chromogenic substrate of β-lactamases, nitrodefin, was measured by the method of Zimmermann and

Rosselet (1977). In this assay, one measures the rate of hydrolysis of a β-lactam by intact cells of gram-negative bacteria. The substrate must first penetrate through the outer membrane in order to reach the enzyme, which is located in the periplasmic space underneath the membrane. By combining Fick's first law of diffusion, which governs the first process, with the Michaelis– Menten equation, which describes the process of enzymatic hydrolysis, one can obtain the permeability coefficient of the outer membrane (Zimmermann and Rosselet, 1977; Nikaido *et al.*, 1983). The results showed (Angus *et al.*, 1982) that the permeability of the *P. aeruginosa* outer membrane to nitrocefin was about 10- to 100-fold lower than the permeability of the *E. coli* outer membrane to other β-lactam antibiotics (see also Nicas and Hancock, 1983a). The same approach was also used by Yoshimura and Nikaido (1982), who compared the permeability of the *P. aeruginosa* and *E. coli* outer membranes to the same set of cephalosporins, cephaloridine and cephacetrile. The results showed that the permeability of the *P. seruginosa* outer membrane to these compounds was 100- to 400-fold lower than that of the *E. coli* outer membranes (see Table II).

A similar methodology was also used to measure the permeability of outer membranes toward phosphorylated compounds, glucose 6-phosphate and *p*-nitrophenyl phosphate, by determining the rates of hydrolysis of these compounds by the periplasmic alkaline phosphatase in intact cells. Again, the permeability of the *P. aeruginosa* outer membrane was about 100- to 200-fold lower than that of the *E. coli* membrane (Yoshimura and Nikaido, 1982).

Finally, the permeability of *P. aeruginosa* outer membrane to a range of carbon sources was estimated from the apparent K_m for the transport of, and growth on, these substrates. These are very rough estimates, and the theoretical limitations of the method are detailed in the original publication (Yoshimura, and Nikaido, 1982). Nevertheless, the K_m values for the transport of fructose, gluconate, 2-ketogluconate, acetate, fumarate, and citrate were uniformly high, in the range 40–150 μM, in contrast to the very low K_m values usually observed in *E. coli*, in most cases less than a few micromolar (Koch, 1971). These values of K_m are consistent with the *P. aeruginosa* outer membrane permeability being lower than that of the *E. coli* membrane by a factor of 10–50. An interesting observation was that the growth K_m for glucose was exceptionally low, lower than 1 μM (Yoshimura and Nikaido, 1982). Presumably this reflects the presence of a glucose-specific channel, created by the protein D1 (see Section IV,B). It should also be mentioned that low K_m values observed for amino acid transport systems (for references see Yoshimura and Nikaido, 1982) are not necessarily inconsistent with low permeability of the outer membrane, because the outer membrane is unlikely to become a rate-limiting step for transport systems with lower V_{max} values (Nikaido and Vaara, 1985).

We emphasize here that the low permeability of the *P. aeruginosa* outer membrane is reflected in the low efficiency of purified *P. aeruginosa* porin in

forming large channels in black lipid bilayers (Benz and Hancock, 1981) and reconstituted proteoliposomes (Yoshimura *et al.*, 1983). For example, in order to produce similar swelling rates in solutions of small sugars, about 40-fold more *P. aeruginosa* porin (by weight) than *E. coli* OmpF porin was needed. If we consider the difference in the size of the pores, it is clear that the proportion of large channels is much smaller than 1 in 40 with the *P. aeruginosa* porin.

Whatever the molecular mechanism that influences the size of the pores, limited attempts to increase the size of the small channels have been unsuccessful. Thus, the majority of pores remain functionally closed to β-lactams even when the cells are grown with limiting amounts of carbon sources in a chemostat, a condition in which opening of the pores might be beneficial for the cell (Yoshimura and Nikaido, 1982). Zimmermann (1980) isolated, after a mutagenesis procedure involving multiple steps, a mutant of *P. aeruginosa* that shows unusually high sensitivity to a number of antibiotics, and Angus *et al.* (1982) have shown that the outer membrane of the mutant has a more than 10-fold higher permeability to nitrocefin. Furthermore, these authors showed that the nitrocefin permeation process has a low temperature coefficient, and is therefore likely to be taking place in water-filled channels, presumably those of protein F. In spite of this, the behavior of the protein F during extraction and gel electrophoresis was apparently unaltered in the mutant. Since significant alterations in the lipopolysaccharide of the mutant, including markedly decreased levels of core components, glucose and rhamnose, as well as an increased level of 2-OH-dodecanoic acid, were demonstrated (Kropinski *et al.*, 1982), it is very tempting to assume that the alteration of the lipopolysaccharide has influenced the proportion of open protein F channels. However, because the mutant was isolated after heavy mutagenesis and in a strain not suitable for genetic analysis, it was difficult to prove a causal relationship between the altered lipopolysaccharide and increased permeability. Angus *et al.* (1982) overcame this difficulty in part by isolating single-step revertants from the mutant. These revertants showed much lower outer membrane permeability, and at the same time produced lipopolysaccharides similar in composition to that of the wild type (Kropinski *et al.*, 1982). Thus, it seems clear that the lipopolysaccharide alteration is indeed responsible for the increased permeability in the mutant, but the molecular mechanism whereby the permeability is altered is not yet fully understood. In fact, proteoliposome reconstitution showed that the protein F isolated from the mutant exhibited permeability similar to that of the wild type, in the presence of either the wild-type or the mutant lipopolysaccharide (H. Nikaido, unpublished observations). It is possible that the proteoliposome assay cannot detect subtle differences in the open/closed states of the pore. In addition, a demonstrated defect in a divalent cation binding site of the lipopolysaccharide of strain Z61 (Peterson *et al.*, 1985) as well as an observed increase in the permeability of Z61 to hydrophobic compounds (B. L. Angus and R. E. W. Hancock, unpublished

observations), considerably complicate analysis of this strain. Thus, more studies are needed for a full understanding of the properties of the channel in the mutant Z61.

D. Physical Properties of Protein F

In *E. coli* and *S. typhimurium*, porins exist as tightly associated trimers which resist dissociation even in SDS, as long as the temperature does not exceed about 70°C (see, e.g., Nakae *et al.*, 1979). In contrast, *P. aeruginosa* porin migrates as monomers in SDS–polyacrylamide electrophoresis, even when solubilized in SDS without heating (Hancock *et al.*, 1979). However, cross-linking studies showed that the protein exists in an oligomeric state in the intact outer membrane (Angus and Hancock, 1983). Thus, treatment of the intact outer membrane with a cleavable cross-linker, dithiobis(succinimidyl propionate) (DTSP), reacting with amino groups, results in the formation of very large amounts of protein F dimers and few heterologous oligomers containing protein F and another protein. This is strong evidence that protein F indeed exists as an oligomer containing at least two monomeric units. The size of the oligomer appeared to be trimeric because cross-linking of the protein F purified in Triton X-100 produced some trimers, although in much smaller amounts than the dimers. As stated by Angus and Hancock (1983), this is a common problem with the cross-linking of many porins: because these proteins have very few accessible amino groups, it sometimes becomes very difficult to produce a complete cross-linking of all subunits. However, in view of the recent identification of dimeric porins, it may be worthwhile to confirm the trimeric nature of protein F by different approaches.

It has been found that oligomeric protein F, purified in Triton X-100, is active in forming channels in proteoliposomes and black lipid film reconstitution systems (Hancock *et al.*, 1979; Benz and Hancock, 1981). More recently, it was shown that the monomeric protein, purified in SDS and confirmed to be monomeric by ultracentrifugation, is capable of forming channels in proteoliposomes (Yoshimura *et al.*, 1983). Although these workers unfortunately did not compare the activity of the monomeric preparation with that of oligomers purified in Triton X-100, the monomers appeared to be nearly fully active, because their activity was as high as 2.5% of that of trimeric *E. coli* porin. An alternative possibility is that the monomers were inactive, and only the rare oligomers that were formed during reconstitution could form channels. Such a hypothesis is attractive because it would also explain the low permeability of the *P. aeruginosa* outer membrane, provided that the monomer–oligomer equilibrium lies far toward the side of the monomers. However, available evidence does not seem to favor this hypothesis. First, in intact outer membranes, a large fraction of protein F appears to exist in an oligomeric state, as revealed by the cross-linking study (Angus and Hancock, 1983). Second, the permeability conferred to proteoliposomes by adding increasing amounts of protein F monomers was perfectly proportional to the amount added (Yoshimura *et al.*, 1983), whereas an upward concave curve might have been expected if only the oligomers formed pores. Third, the pore-forming specific activity was almost identical between the leading edge of the protein F peak eluted from the gel filtration column, i.e., the fraction expected to be enriched in oligomers, and the trailing edge of the same peak (Yoshimura *et al.*, 1983). Fourth, electroeluted monomers of protein F are active in lipid bilayer membranes (Woodruff *et al.*, 1986). Thus, the available evidence seems to suggest strongly that monomer units of protein F can form diffusion channels. We also note that the pore-forming activity of monomeric porins is not an observation limited to protein F. There is now strong evidence that *Rhodopseudomonas sphaeroides* porin is active in its monomeric form (Weckesser *et al.*, 1984), and there are similar, albeit somewhat weaker, data for the *Paracoccus denitrificans* porin (Zalman and Nikaido, 1985).

The monomeric protein F, purified in SDS, was rich in β-sheet structures as determined by circular dichroism (Mizuno and Kageyama, 1979). As the native trimeric porins of *E. coli* and *S. typhimurium* are also rich in β-sheet structure, which is converted into random coil and α-helix upon dissociation and denaturation in hot SDS (Nakamura and Mizushima, 1976; Nakae *et al.*, 1979; Tokunaga *et al.*, 1979), these results suggest that the monomeric protein F retains its nearly native structure even in SDS as long as the temperature remains relatively low, an observation consistent with the strong pore-forming activity of this preparation. What makes the monomers stable against SDS-induced denaturation? One factor is already known. One needs to add SH reagents, such as 2-mercaptoethanol, for the complete denaturation of protein F (Hancock and Carey, 1979), an observation strongly suggesting that disulfide bridges are involved in the stabilization of the tertiary structure of this protein. However, the conversion of presumably nearly native form, migrating at the position of 33,000-dalton protein in SDS–polyacrylamide gel electrophoresis, to the completely denatured from, migrating at the 42,000-dalton position, is apparently a complex process involving several intermediates. It requires the presence of 2-mercaptoethanol as well as denaturation by trichloroacetic acid or by heating. In the latter case, incomplete denaturation and formation of various intermediate forms apparently occurred at lower than optimal concentrations of mercaptoethanol (Hancock and Carey, 1979). It is interesting to note that *R. spaheroides* porin monomers, which are active in forming channels, are also resistant to SDS denaturation, and that complete denaturation of this protein, as indicated by the conversion of β-sheet structure into a mixture of α-helix and random coils, requires either heating for a long period at 100°C in the presence of 2-mercaptoethanol or treatment with trichloroacetic acid (Weckesser *et al.*, 1984).

Protein F apparently interacts with lipopolysaccharides, as revealed by the

formation of cross-linked protein F/lipopolysaccharide products in chemical cross-linking experiments (Angus and Hancock, 1983). It has been proposed that this interaction influences the function of the porin channel. However, certain data argue against this proposal. These data include the demonstration for other porins that lipopolysaccharide is not required for porin function (Parr *et al.*, 1986) and the demonstrated function of protein F preparations electroeluted from gels, and thus apparently uncontaminated by lipopolysaccharide. Nevertheless, the possibility exists that the lipopolysaccharide stabilizes the tertiary and quarternary structures of protein F (Hancock and Carey, 1979), although at present we lack strong evidence for such an effect. Protein F also remains associated with the peptidoglycan layer after the extraction of most other proteins and lipid components with Triton X-100/EDTA or SDS (Hancock *et al.*, 1981a). The physiological implications of this association are unknown, and recently Lugtenberg and van Alphen (1983) argued rather persuasively, at least for enteric bacteria, that such an association is likely to be a laboratory artifact.

Unlike the *E. coli* OmpF porin, which is being studied by X-ray crystallography (Garavito *et al.*, 1983), protein F has not yet been crystallized. The study of its three-dimensional structure has therefore to rely on approaches such as covalent labeling and binding of monoclonal antibodies. Some progress has been made in the latter studies. Thus, several monoclonal antibodies against protein F have been produced, some of which react with the 31,000-dalton fragment of protein F produced by papain and trypsin cleavage, and one of which reacts with cyanogen bromide-cleaved fragment(s) (Mutharia and Hancock, 1985a). It is hoped that this approach will lead to the identification of antibody-binding epitopes on the surface of this protein.

IV. Alternative Porins of *Pseudomonas aeruginosa*

A. Small Anion-Specific Channel Formation by Protein P

When *P. aeruginosa* is grown on medium deficient in phosphate [0.2 m*M* inorganic phosphate (P_i)], a polypeptide, protein P, is produced in high copy number in the outer membrane (Poole and Hancock, 1983). Protein P, like most enterobacterial porins, forms oligomers that resist dissociation even during SDS–polyacrylamide gel electrophoresis when solubilized at temperatures lower than 70°C, but dissociates into monomers of 47,000 daltons when solubilized at higher temperatures (Hancock *et al.*, 1982). Chemical cross-linking with DTSP has suggested that the protein P oligomers are trimers (Angus and Hancock, 1983). However, protein P has two properties which differentiate it from many

porins. First, in chemical cross-linking experiments, internal cross-links in the individual monomer subunits are observed (Angus and Hancock, 1983), a feature so far seen clearly only for the PhoE porin protein (a low phosphate-inducible porin of *E. coli*). Second, the noncovalent interaction of protein P with peptidoglycan is not strong (Hancock *et al.*, 1982), using the usual operational definition (most porins cannot be easily solubilized from the peptidoglycan with detergents and low salt at modest temperatures).

Protein P appears to form part of a phosphate starvation-inducible regulon. When cells are shifted from medium containing moderate concentrations of phosphate (0.6 m*M*) to medium containing a lower concentration of phosphate (0.2 m*M*), the cells continue to grow at preshift rates for about one generation and then shift to a new (lower) growth rate. At this time, they simultaneously start to produce the enzymes alkaline phosphatase and phospholipase C (in both the periplasm and the supernatant), a 37-kDa periplasmic protein, and protein P (Poole and Hancock, 1983). Mutants which hyperproduce all four of these components (Gray *et al.*, 1982; Poole and Hancock, 1984) under phosphate-sufficient conditions, as well as mutants unable to induce any of the four components in response to phosphate starvation (Hancock *et al.*, 1982), have been demonstrated. These data strongly suggest the existence of a *pho* regulon. Furthermore, since heat-stable hemolysin (a glycolipid; Johnson and Boese-Marrazzo, 1980) and a phosphate transport system (LaCoste *et al.*, 1981) are similarly regulated by phosphate starvation, this *pho* regulon may involve a large number of genes. The coregulation of protein P with a number of proteins involved in phosphate acquisition strongly suggests that protein P is involved in phosphate transport across the outer membrane. In agreement with this concept a Tn*501* insertion mutant lacking protein P demonstrated a 10-fold increase in the K_m for high affinity phosphate transport (Poole and Hancock, 1986).

When protein P was purified and incorporated into black lipid bilayer membranes it gave rise to single-step conductance events (Fig. 7) significantly smaller than those of other porins studied to date (Table III). In agreement with these *in vitro* measurements, the induction of protein P *in vivo* did not allow increased uptake across the outer membrane of the β-lactam compound nitrocefin (Poole and Hancock, 1983), which can pass across the outer membrane through the pores of *P. aeruginosa* protein F or *E. coli* porins (Nicas and Hancock, 1983a).

Single-step conductance measurements were performed with a wide variety of salt solutions (Hancock *et al.*, 1982; Benz *et al.*, 1983). The data suggested that the mobile species giving rise to the conductance were anions. For example, even when the cation was large (e.g., $Tris^+$) and the anion small (Cl^-), a single-channel conductance similar to that for the salt K^+Cl^- was observed. In contrast, when the cation was small (K^+) and the anion large ($HEPES^-$), no conductance was observed (Hancock *et al.*, 1982). Zero-current potential measurements fitted to the Goldman–Hodgkin–Katz equation confirmed that the

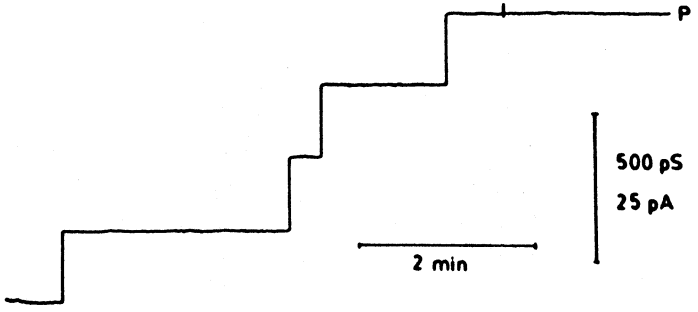

FIG. 7. Stepwise increases in conductance due to the insertion of single channels of protein P into lipid bilayer membranes. The diphytanoylphosphatidylcholine membrane was bathed with 1 *M* KCl and the applied voltage was 50 mV. The conductance increments observed were 20-fold less than those observed in the presence of protein F under similar conditions. pA, Picoampere.

protein P channel exhibited at least 100-fold preference for anions over cations (Benz *et al.*, 1983). By varying the size of the permeating anion and fixing the type of cation (K^+) it was possible to obtain a quite accurate measurement of the effective diameter of the protein P channel (0.6 nm). There was a logarithmic relationship between the single-channel conductance and the anhydrous radius of the permeating anion (Benz *et al.*, 1983). This indicated that the anions were at least partly dehydrated as they entered the channel. Removal of water from the anions would presumably require energy. The energy input for dehydration of anions could conceivably come from binding of the anions to positively charged sites within the channel.

Three pieces of data obtained for the native protein P channel favored the existence of an anion-binding site or sites within the protein P channel. First, the anion selectivity of the channel could be explained by the existence of a positively charged cloud shell within the channel which could attract anions and repel cations. Second, titrating the pH of the salt solution bathing the channel between pH 7 and 9, thus causing potential deprotonation of lysine amino groups, caused a 2.5-fold decrease in single-channel anion conductance (Hancock *et al.*, 1983b) (similar titration experiments between pH 3 and pH 5 also demonstrated within the channel the existence of charged carboxyl groups, which modulated the rate of anion passage but did not strongly influence selectivity). Third, measurement of the single-channel conductance as a function of salt concentration for a variety of salts demonstrated saturation at high salt concentrations for the protein P channel, but a linear relationship for all other porins studied (Fig. 8). The data could be redrawn as an Eadie–Hofstee plot to demonstrate a binding site with K_d values for Cl^-, NO_3^-, $HCOO^-$, CH_3OO^-, and HCO_3^- of 40, 30, 300, 80, and 100 m*M*, respectively (Benz *et al.*, 1983; Benz and Hancock, 1986).

To investigate the actual nature of the anion binding site in the protein P

TABLE III
SINGLE-CHANNEL CONDUCTANCE IN 1*M* KCl AND SELECTIVITY OF BACTERIAL PORIN PROTEINS[a]

Bacterium	Porin	Selectivity at pH 7 (permeability for K^+/ permeability for Cl^-)	Single-channel conductance in 1 *M* KCl (nS)	Estimated pore diameter (nm)[b]
Pseudomonas aeruginosa	P	0.01	0.25	0.6
	F	2.7	5.6	2.0
Escherichia coli	OmpF	3.7[c]	2.1[c]	1.2
	OmpC	13.4[c]	1.5[c]	1.0
	PhoE	0.33[c]	1.8	1.1
	NmpC	0.27[c]	1.7	1.1
	K	15.1[c]	1.8	1.1
Salmonella typhimurium	OmpD (38,000)	22.6[c]	2.4	1.3
	OmpF (39,000)	13.6[c]	2.2	1.2
	OmpC (40,000)	41.0[c]	2.4	1.3
Yersinia pestis	E	12.6[c]	1.4	1.0
Aeromonas salmonicida	42,000	—	1.6	1.1
Neisseria gonorrhoeae	I	0.3	1.4[d]	1.0
N. meningitidis	I	0.8[d]	6.0[d]	2.1

[a] Data from Benz and Hancock (1981), Benz *et al.* (1980, 1982, 1983, 1984), Young *et al.* (1983), Lynch *et al.* (1984), Darveau *et al.* (1983a,b), and Hindahl *et al.* (1984).

[b] Estimated using the formula $\Lambda = \sigma\pi r^2/l$, where Λ is the average single channel conductance, σ the bulk conductivity, r the radius of the channel; and l the length of the channel (assumed to be the width of the membrane, 6 nm). This is a crude estimate, employing certain assumptions, but the result obtained agrees well with other estimates. The diameter of the protein P channel was obtained by determining the maximal-sized anion capable of passing through the channel.

[c] Benz *et al.* (1985).

[d] Data for NaCl, which usually gives lower single-channel conductances and higher anion selectivity than KCl due to the higher hydration and consequent lower mobility of Na^+ ions.

channel, we chemically acetylated available histidine and lysine amino groups with acetic anhydride (Hancock *et al.*, 1983b). The protein remained in its trimeric configuration although slight shifts in the apparent molecular weights of the monomer and trimer form were observed. Three major alterations in measured channel parameters were observed (Table IV). First, the single-channel conductance in 1 *M* KCl decreased 10-fold. Second, the anion selectivity, as measured by zero-current potential measurements, decreased at least 30-fold, such that the acetylated channel was only 3-fold selective for chloride over potassium. Third, there was now an almost linear relationship between salt concentration and single-channel conductance (Fig. 8), indicating that the strong

TABLE IV
PROPERTIES OF NATIVE AND ACETYLATED PROTEIN P[a]

Property	Native protein P	Acetylated protein P
Apparent molecular weight		
Monomer	47,000	50,000
Oligomer	100,000	95,000
Selectivity		
Permeability of Cl^-/permeability of K^+	>100	3
Single-channel conductance (in 1 *M* KCl)	250 pS	25 pS
Dependence of single-channel conductance on salt concentration	Saturates (demonstrates an anion binding site)	Linear (free diffusion)

[a] Data from Hancock *et al.* (1982, 1983b).

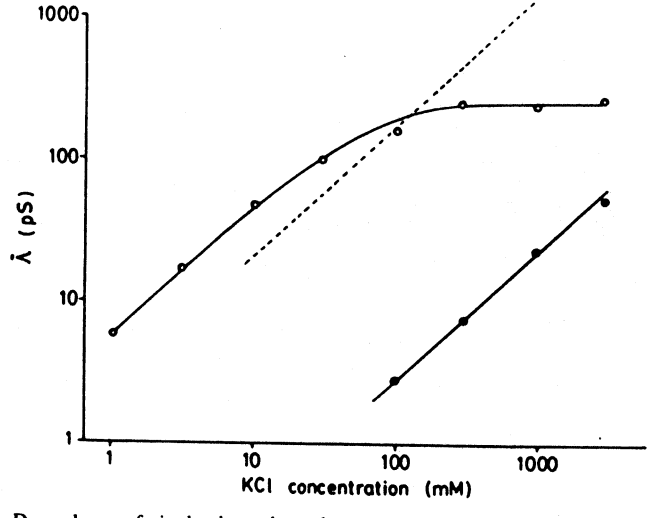

FIG. 8. Dependence of single-channel conductance on the salt concentration of the solution bathing the membrane. The porins used were protein P (open circles), acetylated protein P (closed circles), and the *E. coli* PhoE porin (dashed line). Experimental conditions were as described in the legend of Fig. 6 except that individual points were derived from separate experiments using different salt solutions bathing the membrane. The average single-channel conductance ($\bar{\Lambda}$) was obtained by averaging at least 100 of the step conductance increases observed with each salt (e.g., see Fig. 7). A linear relationship between single-channel conductance and salt concentration typically indicates relatively free diffusion through a water-filled channel, whereas a saturation curve as seen for the native protein P channel indicates the presence of a single anion-binding site.

ion binding site had been lost and ions now diffused relatively freely through the channel.

These results strongly indicate that the basis of the salt concentration-dependent saturation and strong anion selectivity of the protein P channel is the presence of positively charged amino groups within the channel. Both the acetylation with acetic anhydride and the pH dependence of conductance favor the hypothesis that the ε-amino groups of lysines are involved. By measuring the ability of phosphate to competitively block the conductance of chloride ions, protein P was shown to have a high affinity phospate binding site (Hancock and Benz, 1986). Chemical modification experiments, as well as the kinetics of inhibition, suggested that the above-described anion binding site and the phosphate binding site were identical, although the affinity of the site for phosphate was at least 100-fold greater than for other anions.

While these data support the involvement of protein P in phosphate transport, since phosphate anions are small enough to pass through the channel with a single-channel conductance in 1 *M* K_2HPO_4 of 6 pS, they do not explain the theoretical requirement for protein P to mediate specific and unidirectional uptake of phosphate (*in vitro* the channel has no apparent orientation and Cl^- permeates 25 times faster than $H_2PO_4^-$). Thus, a periplasmic phosphate-binding protein (the 37,000 protein) with a high binding affinity (K_d = 1 μ*M*) for phosphate starvation-inducible transport system was sought and discovered (Poole and Hancock, 1984). It is our working hypothesis that this periplasmic phosphate-binding protein is required to make phosphate uptake through the protein P channel unidirectional.

B. PROTEIN D1, A GLUCOSE-INDUCIBLE PORIN

When *P. aeruginosa* is grown on glucose as sole carbon source, a protein with a monomer molecular weight of 46,000 is induced (Hancock and Carey, 1980). Chemical cross-linking studies have suggested that the protein is a trimer in its native state (Angus and Hancock, 1983). However, D1 does not apparently form SDS-resistant oligomers. Instead, increasing the temperature of solubilization in SDS causes the protein to decrease its mobility such that the apparent molecular weight is 35,000 after solubilization at temperatures below 45°C and 46,000 at temperatures above 56°C. Protein D1 was purified and incorporated into synthetic phosphatidylcholine liposomes and shown to make the liposomes leaky for glucose and sucrose but not a 20,000-dalton dextran (Hancock and Carey, 1980). Since protein D1 is coregulated under all growth conditions studied with a high-affinity glucose transport system, including a periplasmic glucose-binding protein, we have suggested that protein D1 is a component of this transport system. Furthermore, many similarities have been noted (Hancock and Carey, 1980) with

the well-studied *E. coli* maltose (glucose disaccharide) transport system, which involves the outer membrane LamB porin protein (Hengge and Boos, 1983).

V. Permeability of *Pseudomonas aeruginosa* to Hydrophobic Compounds

Most gram-negative bacteria exclude all hydrophobic and some amphiphilic compounds (Nikaido, 1976; Nikaido and Nakae, 1979; Hancock, 1984). Such compounds might normally be expected to partition relatively easily into the interior of membranes. However, in most cases, with the possible exception of some *Neisseria* and *Haemophilus* strains (see, e.g., Sarubbi *et al.*, 1975) and of deep rough mutants of *E. coli* and *Salmonella* (Nikaido, 1976) [in the absence of high Mg^{2+} levels (Stan-Lotter *et al.*, 1979)], gram-negative bacteria are quite resistant to hydrophobic antibiotics and do not allow the uptake of hydrophobic compounds such as crystal violet, *N*-phenylnapthylamine (NPN), and 8-anilino-1-naphthalenesulfonic acid (ANS) (Nikaido and Nakae, 1979).

Pseudomonas aeruginosa outer membrane also appears to have a low permeability toward hydrophobic solutes, as judged by its high minimal inhibitory concentrations (MIC) for hydrophobic antibiotics (Table V). Furthermore, intact *P. aeruginosa* cells do not take up NPN or ANS (Loh *et al.*, 1984). Although practically all strains of *P. aeruginosa* (Stanier *et al.*, 1966), including the genetically well-studied strain PAO1 (T. I. Nicas and R. E. W. Hancock, unpublished observations), grow on hexadecane as the sole carbon source, this result is not necessarily contradictory to the generally low permeability of this

TABLE V
MINIMAL INHIBITORY CONCENTRATIONS (MIC) FOR VARIOUS HYDROPHOBIC ANTIBIOTICS OF *Pseudomonas aeruginosa* AND *Escherichia coli*[a]

Antibiotic	*P. aeruginosa*	*E. coli*
Clindamycin	>64	>64
Erythromycin	200	100
Fusidic acid	300	300
Nafcillin	>128	>128
Novobiocin	>128	30
Oxacillin	>128	>128
Rifampicin	16	10
Vancomycin	>64	>64

[a] Data from Angus *et al.* (1982), Fass and Barnisham (1979), Kropinski *et al.* (1979), and Viljanen and Vaara (1984), using a variety of representative strains.

membrane to hydrophobic compounds. As predicted from the physical mechanism of transmembrane diffusion, the penetration rate for extremely hydrophobic compounds will be significantly high even in this type of membrane (Nikaido and Vaara, 1985).

Since, as described in detail below, the uptake of NPN and ANS can be increased by treatment with outer membrane-disorganizing agents such as EDTA (Loh *et al.*, 1984), it may be assumed that the stabilization of the outer surface of the outer membrane by noncovalent cross-bridging of adjacent LPS molecules by divalent cations and by the binding of LPS to proteins prevents lateral spatial displacement of outer membrane components and consequently provides a barrier to the uptake of hydrophobic substances.

VI. Interaction of Aminoglycosides and Polymyxin B with the Outer Membrane of *Pseudomonas aeruginosa*

A. Interaction of Aminoglycosides and Polymyxin B with Lipopolysaccharide

Both polymyxin B and aminoglycosides are polycationic antibiotics; therefore the possibility that these antibiotics would bind to the divalent cation-binding sites on lipopolysaccharide was investigated. Polymyxin B has been shown to bind to *S. typhimurium* lipopolysaccharide at a high-affinity site with a K_d of 0.3 μM (Schindler and Osborn, 1979). To demonstrate binding of polymyxin B to *P. aeruginosa* lipopolysaccharide, two types of experiments were performed. First, polymyxin B was dansylated using the method of Schindler and Teuber (1975). Addition of dansylated polymyxin B to purified lipopolysaccharide (or to intact *P. aeruginosa* cells) caused a blue shift in the fluorescence emission maximum and a dramatic enhancement of fluorescence (Moore *et al.*, 1984). Titration of dansylpolymyxin at constant lipopolysaccharide levels suggested that half maximal binding of dansylpolymyxin occurred at approximately 0.4 μM (Moore *et al.*, 1986). Dansylpolymyxin binding was inhibited by divalent cations (and by aminoglycosides), suggesting the possibility that dansylpolymyxin was binding to divalent cation-binding sites on lipopolysaccharide.

The second method used was competition with the cationic spin label probe CAT_{12}, which binds to a single cation-binding site on *E. coli* (Coughlin *et al.*, 1981) and *P. aeruginosa* (Peterson *et al.*, 1985) lipopolysaccharide. Polymyxin B, the aminoglycosides gentamicin and streptomycin, and Mg^{2+} all competed with CAT_{12} for binding to *P. aeruginosa* lipopolysaccharide, again suggesting that these agents all interact with a divalent cation-binding site on lipopolysaccharide.

B. Permeabilization of Outer Membranes by Aminoglycosides, Polymyxin B, and EDTA

With other gram-negative bacteria it was known that both the polycationic antibiotic polymyxin B and the divalent cation chelator EDTA interact with the lipopolysaccharides of the outer membrane and disrupt the barrier properties of the outer membrane toward lysozyme, hydrophobic antibiotics, and detergents (reviewed in Leive, 1974; Schindler and Teuber, 1975; Storm *et al.*, 1977; Vaara and Vaara, 1981). These results were extended to *P. aeruginosa*. The initial interaction of polymyxin B with *P. aeruginosa* cells was studied by mixing intact cells with dansylpolymyxin (Moore *et al.*, 1984). Cells became heavily surface-labeled with dansylpolymyxin and the fluorescence spectral properties were indistinguishable from those observed upon mixing dansylpolymyxin and purified lipopolysaccharide. Most of the bound dansylpolymyxin cofractionated with outer membranes after cell breakage. These data suggested that the dansylpolymyxin was interacting with cells at lipopolysaccharide sites. It was thus interesting to investigate postinteraction effects.

As mentioned above, the polycationic antibiotic polymyxin B and the divalent cation chelator EDTA are known to permeabilize outer membranes in a variety of bacteria, as judged by a range of direct and indirect assays (reviewed in Hancock, 1984). Three direct assays of outer membrane permeability were used to demonstrate the effects of polymyxin B, EDTA, and polycationic aminoglycosides on the outer membranes of *P. aeruginosa*. As described in Section III,C, the rate of hydrolysis of β-lactams by intact cells provides a direct measurement of outer membrane permeability. Polymyxin, the aminoglycosides gentamicin, streptomycin, and neomycin, and EDTA all increased by 20–30-fold the rate of hydrolysis of the chromogenic β-lactam nitrocefin by periplasmic β-lactamase (controls demonstrated that this increased rate of hydrolysis was not due to β-lactamase release into the supernatant) (Hancock and Wong, 1984). In each case Mg^{2+} antagonized the effects of these agents. Kinetic experiments demonstrated that the rate of nitrocefin hydrolysis was a sigmoidal function of the EDTA concentration, demonstrating that EDTA interacted cooperatively with outer membranes to permeabilize them.

The involvement of a divalent cation-binding site in the outer membrane was demonstrated in the following fashion (Nicas and Hancock, 1983b). Cells were grown with either 0.5 m*M* Ca^{2+} and 0.02 m*M* Mg^{2+} in the medium or with 0.5 m*M* Mg^{2+} and no Ca^{2+}. Atomic absorption studies of cell envelopes demonstrated that cells grown in high Ca^{2+} had preferentially inserted Ca^{2+} into their cell envelopes, whereas cells grown in low Ca^{2+} and high Mg^{2+} had inserted only Mg^{2+}. The outer membranes of both types of cells were permeabilized by EDTA, which can chelate both Mg^{2+} and Ca^{2+}. However, only the Ca^{2+}-grown cells were permeabilized by EGTA, a calcium-specific chelator (again nitrocefin hydrolysis was used as an assay in these experiments). This demonstrates that the specific outer membrane site(s) involved (presumably the lipopolysaccharide divalent cation-binding sites) could be occupied by Ca^{2+} or Mg^{2+}. Other experiments suggested that either Sr^{2+} or Mn^{2+} could substitute for these cations, whereas a variety of other divalent cations could not (Nicas and Hancock, 1983b).

Pseudomonas aeruginosa, like other gram-negative bacteria, does not allow the uptake of hydrophobic compounds, like NPN, because of the properties of its outer membrane (Section V). NPN is a hydrophobic fluorophor that fluoresces strongly in a hydrophobic environment and weakly in an aqueous environment. Thus, NPN fluorescence provides a measurement of the intactness of the hydrophobic permeability barrier. When gentamicin was added in low concentrations (2 μg/ml) to intact *P. aeruginosa* cells in the presence of NPN, under conditions where gentamicin did not kill the cells or become taken up into the cytoplasm (i.e., in the presence of 1 m*M* KCN), fluorescence increased at a low rate initially and then the rate of increase accelerated and finally reached a plateau (Loh *et al.*, 1984) (Fig. 9). When gentamicin was added in higher concentrations to cells, the initial rate of fluorescence enhancement was increased, although the final level of NPN uptake from the medium was almost independent of the NPN concentration. This suggests that increasing concentrations of gentamicin were opening up an increasing number of entry ports for NPN, which was then taken

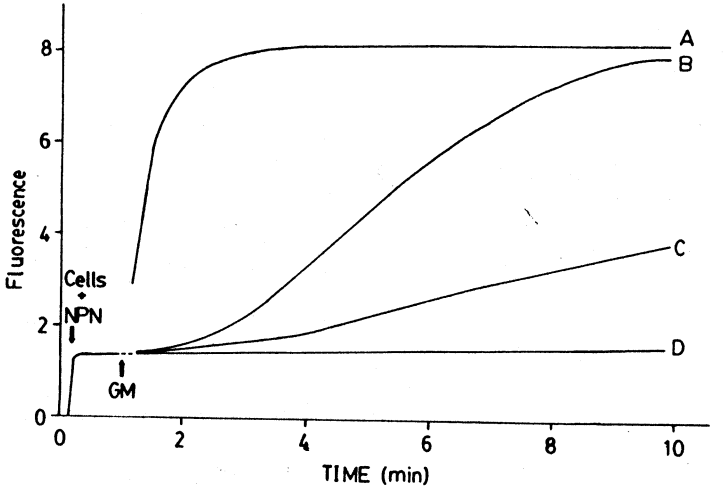

Fig. 9. Time course of increase in NPN fluorescence intensity in the presence of intact *Pseudomonas aeruginosa* cells and different concentrations of gentamicin or Mg^{2+}. At the arrow labeled GM the following additions were made: curve A, 20 μg/ml gentamicin; curve B, 2 μg/ml gentamicin; curve C, 2 μg/ml gentamicin and 100 μ*M* $MgCl_2$; curve D, no gentamicin added (results were identical whether or not $MgCl_2$ was added in the absence of gentamicin). Cells were pretreated with 1 m*M* KCN to prevent postuptake effects of gentamicin. (Reproduced from Loh *et al.*, 1984, with permission.)

up until the membrane hydrophobic core was saturated with NPN. The phenomenon (gentamicin stimulation of NPN fluorescence) was not seen in outer membrane vesicles and thus presumably requires an intact outer membrane surface. A plot of the initial rate of fluorescence increase as a function of gentamicin concentration was sigmoidal. Replotting this as a Hill plot demonstrated that gentamicin interacts with outer membranes in a cooperative fashion with a Hill coefficient of interaction (minimal number of sites involved in the interaction) of around 2. Essentially identical data were obtained for seven other aminoglycoside antibiotics, and the average Hill coefficient for all aminoglycosides was 2.26 ± 0.26. The *Y* axis intercepts of the Hill Plots provided an estimate for a pseudo-association constant. There was a significant [$p < .001$; 29 degrees of freedom (d.f.)] relationship between the logarithm of the pseudo-association constant and the logarithm of the minimal inhibitory concentration for the respective aminoglycoside antibiotics, suggesting that this outer membrane interaction may well be one of the rate-limiting steps in killing of *P. aeruginosa* cells by aminoglycosides.

Both polymyxin B and EDTA also caused an enhancement of NPN fluorescence in intact *P. aeruginosa* cells (Hancock and Wong, 1984). For these agents, and for the aminoglycosides above, Mg^{2+} (and, when tested, Ca^{2+}) antagonized their ability to promote NPN uptake. For gentamicin-promoted NPN uptake, it was shown that antagonism by both Mg^{2+} and Ca^{2+} was dependent on the divalent cation concentration (Loh *et al.*, 1984). These data thus favor the hypothesis that the observed effects occur at an Mg^{2+}-binding site on the outer membrane.

The third method used to specifically demonstrate permeabilization of outer membranes involved the enzyme lysozyme (Hancock *et al.*, 1981b). This 14,000-dalton enzyme is normally excluded by the outer membrane, but when the outer membrane is breached lysozyme can attack its target, the peptidoglycan, and cause cellular lysis. Gentamicin, streptomycin (Hancock *et al.*, 1981b), neomycin, polymyxin B, and EDTA (Hancock and Wong, 1984) all caused rapid lysis of cells in the presence of lysozyme. Inhibitors or mutants preventing the uptake of, or killing of cells by, aminoglycosides did not prevent aminoglycoside-promoted lysozyme lysis, suggesting that this was an early event in the action of aminoglycosides on cells and independent of postuptake effects of these aminoglycosides. The antagonist, Mg^{2+}, inhibited the lysis of cells by lysozyme after treatment with all of the above agents.

C. Resistance to Polymyxin B and Aminoglycosides due to Outer Membrane Alterations

Brown and Melling (1969) demonstrated that cells grown in Mg^{2+}-deficient medium (provided it was also deficient in Ca^{2+}, Sr^{2+}, and Mn^{2+}) were resistant to polymyxin B and EDTA. Nicas and Hancock (1980) isolated two

polymyxin B-resistant mutants and demonstrated that they mimicked the above adaptive resistance in every aspect studied. The observed phenotypic changes were as follows: resistance to killing and lysis by polycationic polymyxins, resistance to killing by polycationic aminoglycosides, resistance to the divalent cation chelator EDTA, altered aminoglycoside uptake kinetics, and a substantial (up to 20-fold) increase in outer membrane protein H1 and corresponding decrease in cell envelope Mg^{2+} levels (Nicas and Hancock, 1980, 1983b; Hancock *et al.*, 1982). Both single-step, spontaneous revertants of the polymyxin B-resistant mutants and adapted strains shifted to Mg^{2+}-rich medium regained all wild-type properties, suggesting a single major alteration in the mutants and adapted cells. The mutants had normal outer membrane permeability (Nicas and Hancock, 1983a) via the hydrophilic (porin) uptake route (as assessed by the nitrocefin method), had no significant changes in fatty acid composition, with the exception of a small decrease in dodecanoic acid, and had no detectable alterations in the levels of lipopolysaccharide or of any phospholipids (Moore *et al.*, 1984). Since the protein H1 increase is associated with a decrease in Mg^{2+} levels in the cell envelope, Nicas and Hancock (1980) proposed that protein H1 associates with the lipopolysaccharide and functionally replaces Mg^{2+}, thus shielding the lipopolysaccharide Mg^{2+}-binding site from attack by polymyxin B, gentamicin, and EDTA.

D. Self-Promoted Uptake Hypothesis

The above data clearly demonstrate that aminoglycosides and polymyxin B interact with the outer membrane, probably at surface sites where divalent cations cross-bridge adjacent lipopolysaccharide molecules. The competitive displacement of these divalent cations by the polycationic antibiotics allows uptake of the β-lactam nitrocefin, the protein lysozyme, and the hydrophobic fluorophor NPN. In agreement with this concept, the removal of Mg^{2+} from outer membrane sites by chelation with EDTA causes similar permeabilization events to occur, and all of these outer membrane permeabilizing activities are antagonized (presumably due to competition) by Mg^{2+}.

Thus, Hancock *et al.* (1982; Hancock, 1981) have proposed that aminoglycosides (and possibly polymyxin) act at these outer membrane sites in order to promote their own uptake, although direct measurement of aminoglycoside uptake across the outer membrane has not been possible to date. This extends to other cationic compounds, notably aminoglycosides, what was generally believed to be the mode of penetration of polymyxin across the *E. coli* outer membrane. Three pieces of data support this so-called self-promoted uptake model, and suggest the self-promoted uptake is an important step in the killing of *P. aeruginosa* by aminoglycosides. First, divalent cations have been known for years to strongly antagonize the uptake of (Bryan and van den Elzen, 1976) and killing by (Zimelis and Jackson, 1973) aminoglycosides in *P. aeruginosa;* these

data can be explained in part by competition for the divalent cation-binding site involved in self-promoted uptake (Nicas and Hancock, 1983b). Second, as noted above, a significant correlation between the outer membrane permeabilizing effects of different aminoglycosides (using NPN as a probe) and the minimal inhibitory concentrations for these aminoglycosides was observed (Loh *et al.*, 1984). Third, when the Mg^{2+} levels in the cell envelope were decreased by displacement with protein H1 under certain growth conditions or in specific mutants, the cells became cross-resistant to polymyxin B, aminoglycosides, and EDTA (Nicas and Hancock, 1980, 1983b) and, in the one case studied, demonstrated altered uptake kinetics for streptomycin (Hancock *et al.*, 1982). This, together with the lack of alteration in the porin-mediated hydrophilic uptake route in the outer membranes of these mutants (Nicas and Hancock, 1983a), suggests that self-promoted uptake is a valid mechanism by which polycations can cross the outer membrane of *P. aeruginosa* (and other organisms—see Hancock, 1984, for review).

E. Other Mechanisms of Polymyxin B Resistance

Growth of wild-type cells or the above mutants (Section VI,C) in increasing levels of polymyxin B allows adaptation to resistance to higher levels of polymyxin B (Brown and Watkins, 1970; Gilleland and Conrad, 1982). Associated with this polymyxin B adaptation are multiple phenotypic changes in the adapted strains (see, e.g., Gilleland and Conrad, 1982). We and others have provided evidence that this adaptation involves, in part, formation of a stable state in which polymyxin B is incorporated into the outer membrane but is blocked from further uptake (Traub, 1982; Moore *et al.*, 1984).

VII. Compounds Which Alter Outer Membrane Permeability in *Pseudomonas aeruginosa*

A variety of compounds, as described in Section VI,B, were shown to increase the permeability of the *P. aeruginosa* outer membranes to the β-lactam nitrocefin the hydrophobic fluorophor NPN, and the protein lysozyme. These included eight aminoglycosides, polymyxin B, and EDTA. A broader screen was performed (Hancock and Wong, 1984) using the assay systems described above. A further eight compounds were identified and shown to fit into four broad chemical groupings. Three were polycationic compounds (like the aminoglycosides and polymyxin B), namely poly(L-lysine), polyornithine, and gramicidin S, one was a chelator like EDTA, namely nitrilotriacetate, and two, Tris and cetyltrimethylammonium bromide, were monovalent organic cations. The fourth group

of compounds, including ascorbate and acetyl salicylate, were probably acting as either reducing agents or weak chelators and, unlike the other agents described above, specifically enhanced the rate of nitrocefin permeation but only slightly enhanced lysozyme and NPN uptake. In most cases Mg^{2+} antagonized the effects of these compounds on *P. aeruginosa* outer membrane permeability.

The simplest hypothesis to explain these data is that all of these compounds interact with *P. aeruginosa* outer membranes in a manner reminiscent of the actions of the polycationic antibiotics or of the chelator EDTA, as described in detail in Section VI. However, the data obtained were quite complex (Hancock and Wong, 1984), and while some of this complexity may be explained on the basis of differing affinities of the compounds for outer membrane sites, it cannot be excluded that more than one kind of site is involved (see also Moore *et al.*, 1986). Nevertheless, since the low outer membrane permeability of *P. aeruginosa* apparently influences the outcome of antibiotic therapy (Section III), compounds which increase outer membrane permeability may well prove synergistic with antibiotics. In principle, this has been already demonstrated for some of the compounds described in Sections VI and VII (Rawal *et al.*, 1974; Sykes and Morris, 1975).

VIII. Outer Membranes and Protein Secretion

Pseudomonas aeruginosa has an important property which distinguishes it from certain other gram-negative bacteria including *E. coli*. It is capable of excreting a variety of soluble proteins into the external environment. These proteins include exotoxin A, exoenzyme S, alkaline protease, elastase, phospholipase C, and alkaline phosphatase, as reviewed elsewhere in this volume (Nicas and Iglewski, Chapter 5). Despite this interesting trait and the importance of some of these enzymes in the pathogenesis of *P. aeruginosa* infections, few studies have been addressed to the mechanism of protein excretion across the outer membrane. In contrast, the mechanism of protein secretion across the cytoplasmic membrane, at least in *E. coli*, is relatively well understood (Randall and Hardy, 1984).

The excretion of phospholipase C and/or alkaline phosphatase has been investigated by three groups (Ingram *et al.*, 1973; Stinson and Hayden, 1979; Poole and Hancock, 1983). Unfortunately, two of these studies involved the growth of cells in Tris, which is now known (Irvin *et al.*, 1981; Hancock and Wong, 1984) to be an outer membrane-active compound which alters the permeability of the outer membrane and causes release of lipopolysaccharide from cells. Poole and Hancock (1983) demonstrated that when cells were shifted from phosphate-sufficient to phosphate-deficient medium, alkaline phosphatase and phospholipase C were induced after a lag of approximately one generation. These two

enzymes appeared at approximately the same time both in the periplasm [as functionally defined using the method of Hoshino and Kageyama (1980)] and free in the supernatant. Unfortunately, no pulse chase experiments were performed, so it was impossible to say if the enzymes were first secreted into the periplasm and thereafter excreted through the outer membrane into the supernatant.

At the time of excretion of these enzymes from the cell, the outer membrane did not become overtly leaky as judged by the retention in the periplasm of an enzyme, β-lactamase, and a phosphate-binding protein. In addition, outer membrane permeability, as assessed with nitrocefin, actually decreased slightly. Thus, excretion of the enzymes phospholipase C and alkaline phosphatase does not require outer membrane breakage. While the actual mechanism of protein excretion remains obscure, the suggestion (Bhatti and Ingram, 1982) that alkaline phosphatase is cosecreted with lipopolysaccharide does not appear to be true in all cases, since, at least in one set of experiments, no lipopolysaccharide was found in 50-fold concentrated supernatants containing substantial amounts of alkaline phosphatase and phospholipase C (Poole and Hancock, 1984). Overall, the data suggest that excretion across the outer membrane of alkaline phosphatase and phospholipase C involves a specific mechanism.

IX. Outer Membranes of Pseudomonads Other than *Pseudomonas aeruginosa*

Relatively little information is available on the composition, structure, or functions of outer membranes of other *Pseudomonas* species. Lipopolysaccharide is probably the only component that has been investigated in a somewhat systematic manner. The composition of lipid A and the core oligosaccharide seems to be very similar among most *Pseudomonas* species, with a few exceptions (Wilkinson *et al.*, 1973; Mutharia *et al.*, 1984). *Pseudomonas diminuta* lacks glucosamine, and *P. rubescens* lacks KDO and contains fatty acids with exclusively odd-numbered carbon chains in lipid A. These results are consistent with taxonomic studies which showed that *P. diminuta* is not closely related to most fluorescent and nonfluorescent *Pseudomonas* species (Palleroni *et al.*, 1972) and that "*P. rubescens*" actually does not belong to the genus *Pseudomonas* because of the low GC content in its DNA and its fermentative metabolism (Doudoroff and Palleroni, 1974; Bergan, 1981).

All of the pseudomonads that appear to be closely taxonomically related to *P. aeruginosa* share an antigenic epitope that is present on the outer membrane lipoprotein H2 (Mizuno, 1979; Mutharia and Hancock, 1985b), although these strains have distinct major outer membrane protein patterns on SDS–polyacrylamide gel electrophoresis (Nakajima *et al.*, 1983; L. M. Mutharia, unpublished observations).

It is also possible that the exceptionally low hydrophilic permeability of the *P. aeruginosa* outer membrane is not shared by all other members of the genus *Pseudomonas*. Although some species, such as *P. maltophila* and *P. cepacia*, show resistance to a wide variety of antibiotics and are appearing increasingly in hospital-acquired infections, other species do not show the same degree of resistance to antibiotics. *Pseudomonas fluorescens, P. putida, P. stutzeri,* and *P. alcaligenes* are all described as sensitive to tetracycline, sulfonamides, and trimethoprim (Bergan, 1981). Thus, possibly the porin channels in these latter species are not as inactive as in *P. aeruginosa*. In a preliminary study, fragments of outer membrane of *P. syringae* reconstituted into proteoliposomes showed much higher permeability than those containing the *P. aeruginosa* porin (J. Weckesser, R. E. Hurlbert, and H. Nikaido, unpublished observations). Interestingly, both *P. putida* and *P. syringae* outer membranes contain a protein which interacts with one class of monoclonal antibodies specific for protein F (Mutharia and Hancock, 1985a).

X. Conclusions

In this chapter, we have tried to describe the structure and permeability of the *P. aeruginosa* outer membrane as a rather extreme case among the gram-negative outer membranes. It seems probable that the lipopolysaccharide molecules are very tightly packed together because of the divalent cation bridging of neighboring molecules through the unusually large number of phosphate residues present. This could contribute to the resistance of the outer membrane of this species toward detergent-induced disorganization and to its lack of permeability toward hydrophobic antibiotics. Presumably, the resistance of this species to many hydrophilic antibiotics is due primarily to the low permeability of the porin channel, caused by the small size of most of the channels. In this sense, the *P. aeruginosa* outer membrane is built on the same principles used by other gram-negative species, but subtle changes in construction details have succeeded in producing an outer membrane that acts as an unusually effective permeation barrier.

It now seems very likely that most of gram-negative chemoheterotrophic bacteria, including *P. aeruginosa*, are descendants of a group of photosynthetic, nonsulfur purple bacteria (Fox *et al.*, 1980). In this light, it is interesting that two of these photosynthetic bacteria which have been investigated, *Rhodopseudomonas capsulata* (Flamann and Weckesser, 1984) and *R. sphaeroides* (Weckesser *et al.*, 1984), produce porins with wide channels, which appear to be mostly "open." Presumably these wide, open channels are optimal for the effective uptake of nutrients from the dilute, natural waters in which they live. The much narrower channels could have been a more recent development, such as those found in the Enterobacteriaceae, undoubtedly in response to their spe-

cialized ecological niche, which contains high concentrations of inhibitors, notably bile salts. In addition, other modifications in porin structure must have occurred in different branches of the phylogenetic tree. Perhaps *P. aeruginosa* (and others) developed a way to decrease the size of most of their porin channels and thereby became resistant to various inhibitors, a development that now allows them to flourish in animal bodies as well as in soil and natural waters. The large pore would allow the uptake of nutrients of fairly large size, and at the same time the low permeability would protect the bacteria from inhibitory agents such as bile salts and fatty acids. We do not know, however, why it is necessary for these bacteria to produce so many porin molecules with small channels. Perhaps the protein plays a structural role. In keeping with this possibility, a porin-deficient mutant of *P. aeruginosa* grew poorly unless stabilized by high (0.1 *M*) salt (Nicas and Hancock, 1983a). It is, of course, possible that the organism can produce more porins with large channels under certain environmental conditions hitherto untested.

Although the low permeability of the outer membrane is necessary to make the cells resistant to antibiotics, this is not a sufficient condition in many cases, because even small numbers of antibiotic molecules entering the cell could sometimes be lethal. Thus, in many situations enzymatic inactivation of antibiotics also plays an important role. In this connection, it is interesting to note that some antibiotics are known, or appear, to be inactivated by mechanisms coded by *P. aeruginosa* chromosomal genes (Bryan, 1979). Although in some cases it has been stated that there was no evidence for enzymatic inactivation of antibiotics, we emphasize that even an exceedingly low enzymatic activity will suffice in the presence of a strong outer membrane barrier, as only a few molecules of antibiotic per unit time will trickle through into the periplasmic space. In this sense, the situation may be similar to that in *Enterobacter cloacae,* which shows high-level resistance to cephalosporins hitherto believed to be totally resistant to enzymatic hydrolysis. Recently, it was shown that this organism also produces outer membrane of low permeability, and that the chromosomally determined cephalosporinase (β-lactamase) does hydrolyze these compounds, albeit at a very low rate (Vu and Nikaido, 1985). Calculation showed that even this low level of enzyme activity, which has previously been considered insignificant, suffices to hydrolyze the few cephalosporin molecules that manage to penetrate through the outer membrane. Similar observations were subsequently made with *P. aeruginosa* mutants with derepressed β-lactamase (Bayer *et al.*, 1986). Thus, achieving very high levels of resistance often requires the combination of outer membrane barrier plus periplasmic inactivation, or exclusion from the cytoplasm, of antibiotics. It can be misleading to consider the significance of the outer membrane barrier without considering the latter processes. It is hoped that more careful studies will be undertaken to understand the fate of the antiobiotic molecules after their passage through the *P. aeruginosa* outer membrane.

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