CHAPTER 5

Resistance to Antibacterial Agents Acting on Cell Membranes

Robert E. W. Hancock
Thalia I. Nicas
Department of Microbiology
University of British Columbia
Vancouver, British Columbia, Canada

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I. INTRODUCTION

A. Structure of Bacterial Membranes

Bacterial membranes can be broadly classed into three major types according to structure, function, and cellular location. These are the outer membrane, which is found only in gram-negative bacteria, the cytoplasmic membrane found in all bacteria, and intracytoplasmic membranes which are prominent in some classes of bacteria such as *Rhodopseudomonas* (Cohen and Kaplan, 1981). This chapter will discuss the outer and cytoplasmic membranes only. A general structure for a gram-negative cell envelope is demonstrated in Fig. 1. Gram-positive cells have a different cell envelope structure that completely lacks outer membranes and usually has a thicker peptidoglycan.

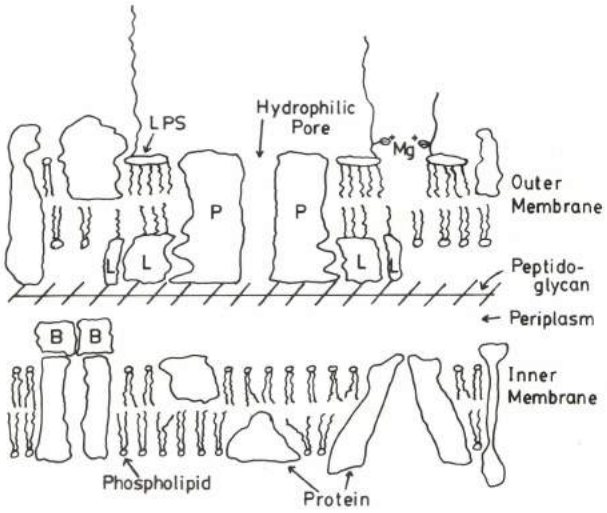

Fig. 1. Schematic diagram of the cell envelope of gram-negative bacteria. The outer membrane structure is modeled from data obtained from our laboratory on *Pseudomonas aeruginosa*. The labeled proteins are porins (P), lipoproteins (L, which in *P. aeruginosa* are noncovalently associated with the peptidoglycan but in enteric organisms are partly covalently attached), and periplasmic substrate binding proteins (B). Significant features of this diagram stressed in the text are (1) LPS as the major cell surface lipidic molecule, (2) cross-bridging of adjacent LPS molecules by Mg^{2+}, (3) the presence of hydrophilic channels of defined exclusion limit formed by outer membrane porins, and (4) the lack of porin channels and presence of lipid bilayer (as opposed to LPS : lipid bilayer) in the inner (cytoplasmic) membrane.

The cytoplasmic membrane is a lipid (usually phospholipid) bilayer membrane liberally studded with a wide variety of polypeptides. Due to the properties of lipid bilayers, the membrane offers a unique cellular compartment, with a hydrophobic interior consisting of the fatty acyl chains of the lipids and hydrophilic cytoplasmic and exterior surfaces provided by the head groups of the lipids. Membranes are dynamic structures containing at least 40% fluid lipids, which are capable of lateral movement at quite rapid rates (several micrometers per second), as well as gel-like domains of lipids, which are quite immobile. The proper functioning of membrane proteins and the ability of molecules to insert themselves into the bilayer are dependent on the presence of fluid lipids. The major functions of cytoplasmic membrane proteins are energy generation [e.g., electron-transport chain carrier proteins and (Ca^{2+},Mg^{2+})-stimulated ATPase], active and facilitated transport of nutrients and export of toxic by-products, and enzymatic synthesis of cell envelope components.

The outer membrane is unusual in that one of the two monolayers [the outer (surface) leaflet] of the bilayer contains an unusual lipidic molecule, lipopolysaccharide (LPS), whereas the inner leaflet contains phospholipids (Muhlradt and Golecki, 1975; Kamio and Nikaido, 1976). What makes LPS unusual is its unique hydrophobic region (lipid A or endotoxin), which generally contains five or six fatty acids linked to diglucosamine, as well as a large carbohydrate chain (oligosaccharide core ± O Antigen) covalently bound to the lipid A region (Luderitz *et al.*, 1982). The LPS carries a net negative charge, resulting in the strong negative surface charge of gram-negative cells (Sherbert and Lakshmi, 1973). One of the most important features of LPS is that it appears to be anchored in the outer membrane by binding to outer membrane proteins (Datta *et al.*, 1977; Mutoh *et al.*, 1978) and by noncovalent cross-bridging of adjacent LPS molecules with divalent cations (Leive *et al.*, 1968; DePamphilis, 1971). Thus, treatment of gram-negative cells with EDTA generally results in removal, by chelation, of the divalent cations and consequent disruption of the outer membrane (see below). In the absence of such chelators, however, the combination of negative charge and divalent cation cross-bridging of LPS provides gram-negative cells with many of their important properties, including resistance to hydrophobic antibiotics, bile salts, detergents, proteases, lipases, and lysozyme (Leive, 1974; Nikaido, 1976; van Alphen *et al.*, 1977). The outer membrane also contains a small number of so-called major proteins present in high copy number (10^5 copies/cell). Some of these proteins anchor the outer membrane to the underlying peptidoglycan, while others (porins) provide water-filled pores for the uptake of hydrophilic nutrients and antibiotics smaller than a given

exclusion limit [which varies according to the bacterium (Nikaido and Nakae, 1979; Benz and Hancock, 1981)].

B. The Membrane as a Target for Antibacterial Agents

The five major functions of lipids are that (1) their surfaces accommodate proteins; (2) they separate aqueous environments, allowing vectorial processes to occur; (3) they provide hydrophobic environments for, for example, the synthesis of hydrophobic compounds; (4) they are required for membrane bilayer formation; and (5) at least in some cases, they appear to modulate the functions of membrane proteins (Sandermann, 1978). As discussed below, the most common target of antibacterials affecting the cytoplasmic membrane is function 2, the property of a membrane as a permeability barrier. Compounds that disrupt the permeability barrier are often hydrophobic compounds which intercalate the lipid bilayer.

Outer membranes are susceptible to a very specific group of polycationic compounds that attack the divalent cation cross-bridges between LPS molecules (by competing for cation binding sites), causing permeabilization of outer membranes to hydrophobic substances and proteins (see Section III,C). Alternatively, outer membranes actually inhibit, by exclusion, the action of such cytoplasmic membrane-active agents as ionophores (see Section IV,A).

II. MEDICAL USEFULNESS OF MEMBRANE-ACTIVE AGENTS

Membrane-active agents include some of the most widely used and effective disinfectants. Antibiotics active on membranes, however, generally show little selectivity in their toxicity to bacterial and mammalian cells. Consequently, their chemotherapeutic usefulness is very limited. Only the polymyxins, which until the discovery of gentamicin and carbenicillin were the only antibiotics effective against *Pseudomonas aeruginosa,* are considered useful in the treatment of infections. As polymyxins are nephrotoxic and can be neurotoxic, they have been replaced with newer antipseudomonal antibiotics for systemic infections. Topical therapy with polymyxins is still useful in the treatment of eye and skin infections, and they are common ingredients in disinfectant creams. In addition, polymyxin B can act synergistically with other antibiotics and has

been used in antibiotic combinations to treat infections with multiply antibiotic-resistant *Serratia marcescens* and *P. cepacia*. Polymyxins have been largely free from the problem of emergence of resistance in formerly susceptible bacteria. However, adaptation to growth in high concentrations has been shown for a number of bacteria (see Section IV,C,4).

Perhaps the major application of membrane-active agents is as tools in the study of membrane functions. The peptide ionophores, uncouplers, and membrane-active bacteriocins have all been useful in this role.

III. MODE OF ACTION OF MEMBRANE-ACTIVE AGENTS

A. Techniques for Examining Antimembrane Activity

A wide variety of techniques are applicable to examining antimembrane agents. They broadly fit into three major categories: (1) techniques for measuring alterations in membrane integrity (permeability), (2) measurements of the activity of membrane-bound enzymes, and (3) methods for studying membrane perturbation.

For the measurement of cytoplasmic membrane integrity, the most commonly used assay (Newton, 1953) is release of A_{260}-adsorbing material (purine and pyrimidine nucleotides). Other useful assays include release of ^{42}K from preloaded cells (Rosenthal *et al.*, 1977), release of cytoplasmic enzymes like β-galactosidase (Gupta, 1975), and release of cellular nitrogen or phosphorous into the medium (Mohan *et al.*, 1962). Of course, the most dramatic indicator of loss of membrane integrity is cell lysis (Brown and Melling, 1969). For measurements of outer membrane integrity, other methods must be used, including release of periplasmic marker enzymes, e.g., alkaline phosphatase and β-lactamase, into the medium (Cerny and Teuber, 1971). In addition, outer membrane permeability can be measured simply and precisely using the hydrolysis of a chromogenic β-lactam, nitrocefin, by periplasmic β-lactamase (Hancock *et al.*, 1981; Nicas and Hancock, 1983a). Enhanced susceptibility to hydrophobic antibiotics like actinomycin D (Leive, 1974), sensitivity to the enzyme lysozyme, which acts on the normally inaccessible peptidoglycan and leads to cell lysis (Repaske, 1958), and enhanced uptake of hydrophobic fluorescent probes (Newton, 1954; Rosenthal *et al.*, 1976) are other effective methods of measuring outer membrane integrity. Alternatively, outer membrane damage in the form of blebbling can easily be seen with an electron microscope (Gilleland *et al.*, 1974). Cell-free model membrane

systems are often used as "simple" probes of membrane-active antibiotic activity (Storm *et al.*, 1977).

In the second category of techniques, i.e., alteration of membrane-bound enzyme activities, one of the preferred enzyme systems for study is respiration or electron transport. This can be measured, using an oxygen electrode, by the disappearance of oxygen from the medium (Teuber, 1974; La Porte *et al.*, 1977). Alternative enzymes that may be studied include (Ca^{2+},Mg^{2+})-stimulated ATPase activity and membrane-transport activity (Teuber, 1974; Levin and Freese, 1977) for the cytoplasmic membrane and phospholipase A1 activity in the outer membrane (Hardaway and Buller, 1979).

As stated above, membrane fluidity is an important property of membranes. Thus, membrane perturbation may be studied by examining membrane fluidity through the use of sophisticated techniques such as electron spin resonance, nuclear magnetic resonance, differential scanning calorimetry, and fluorescence (Pache *et al.*, 1972; London and Feigenson, 1981).

B. Ion Leakage

Several cyclic antibiotics, including valinomycin (a cyclic peptide), nonactin, and monactin (macrocyclic tetralides), function as carriers of K^+ (and to a much lesser extent Na^+) across membranes (Bakker, 1979; Edwards, 1980). They form complexes (1 : 1) with K^+ and shuttle them across the cytoplasmic membrane according to the gradient of K^+ concentration (i.e., $[K^+]_{in} - [K^+]_{out}$) and the protonmotive force (the electrical potential gradient component, $\Delta\psi$). This affects energization of cells as well as many cellular processes, including protein synthesis, which seem to require the high cytoplasmic K^+ concentrations normally maintained in bacterial cells. Such antibiotics carry the general name ionophores.

Other ionophores are linear polyethers (also called linear macrotetralides) such as nonensin, nigericin, A23187, and a number of others (Bakker, 1979; Edwards, 1980). Like the cyclic ionophores, they form complexes with ions. The ion is coordinated by hydrogen bonding with the interior of the molecule that wraps around the ion so that the exterior aspect of the molecule is hydrophobic (i.e., membrane soluble). Nigericin has been shown to mediate the 1 : 1 exchange of K^+ for H^+ across the cytoplasmic membrane (Kaback, 1976), thus eliminating part of the protonmotive force (the pH gradient, ΔpH). The antibiotic A23187, in contrast, is primarily a divalent cation ionophore, with a marked preference for Ca^{2+} and Mg^{2+} (Wulf and Pohl, 1977). The precise details of how these ionophores cause bacterial death are unknown.

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A wide range of chemicals called uncouplers, including dinitrophenol and carbonylcyanide *m*-chlorophenylhydrazone (CCCP), are thought to act as proton ionophores (Kaback, 1976), shuttling protons across the cytoplasmic membrane to neutralize the protonmotive force (see Kaback, 1976, for review of the protonmotive force). This results in the deenergization of cells and consequent bacteriostasis. Other chemicals, including acetate, propionate (Kaback, 1976), and fluoride ions (Eisenberg and Marquis, 1981), act as lipid-soluble proton carriers and neutralize the ΔpH of the protonmotive force.

In addition to these ionophores, a linear peptide of 15 amino acids, gramicidin A, forms channels across the cytoplasmic membrane, making it permeable to alkali ions such as K^+ and Na^+. It is thought that two gramicidin A molecules together form a helical structure with a hydrophobic exterior and a water-filled, negatively charged interior (Bakker, 1979; Edwards, 1980). Thus, gramicidin A is a channel- or pore-forming antibiotic rather than an ionophore like valinomycin; since both antibiotics cause K^+ leakage, however, their biological effects on bacterial cells are very similar.

Bacteriocins are proteins produced by given species of bacteria and are usually able to act against only the producing, or closely related, species (Hardy, 1975). One group of bacteriocins, typified by colicins K, E1, and Ia, has dramatic effects on cellular energy metabolism and subsequently on macromolecular synthesis, leading rapidly to cell death (Hardy, 1975). These colicins were shown to form ion-permeable channels in lipid bilayer membranes (Schein *et al.*, 1978), although unlike the above antibiotics, the colicin channel is able to mediate passage of both cations and anions. It has been suggested that the incorporation of just a single colicin molecule into the cytoplasmic membrane of *Escherichia coli* would deplete the cell of potassium, magnesium, and other small ions within a few seconds (Schein *et al.*, 1978).

C. Cytoplasmic Membrane Disruption

A wide variety of compounds are known to disrupt the cytoplasmic membrane, although in no case is the actual mechanism of disruption understood. Since membrane integrity is a critical feature of living organisms, such agents are usually bactericidal and give rise to rapid cell death. For the phenolic antiseptic, hexachlorophrene, it has been suggested that despite the wide range of different effects on cell membranes, the primary mode of inhibition of bacterial growth is through substrate transport inhibition due to elimination of the protonmotive force (Levin and Freese, 1977). Other lipophilic acids such as fatty acids, which are

inhibitors of some gram-positive cells (Sheu *et al.*, 1972; Levin and Freese, 1977), are thought to act similarly. Alternative agents thought to cause disruption of the cytoplasmic membrane include quaternary ammonium antiseptics such as cetyl trimethylammonium bromide (Cetrimide and Savlon) (Gale and Taylor, 1947; Salton, 1951), cyclic polypeptides like tyrocidin A and gramicidin S (Pache *et al.*, 1972; Edwards, 1980), and cyclic polypeptides with a hydrophobic tail (Newton, 1953; Mohan *et al.*, 1962; Teuber, 1974) including polymyxin B, colistin methosulfonate, and octapeptin (EM49). Polymyxin B and octapeptin cause H^+ and K^+ leakage across the cytoplasmic membrane at concentrations around the MIC (Rosenthal *et al.*, 1977). Some experiments, in which polymyxin B or octapeptin were immobilized on Sephadex beads, have suggested that such ion leakage across the cytoplasmic membrane can be caused by the binding of the antibiotics to the outer membrane (La Porte *et al.*, 1977).

D. Outer Membrane Disruption

It has been proposed that polycationic antibiotics displace Mg^{2+} from surface anionic sites (phosphate or 2-keto-3-deoxyoctanate) on the LPS (Nicas and Hancock, 1980; Hancock, 1981; see Fig. 1). The bridging by Mg^{2+} of such sites on adjacent LPS molecules is important for outer membrane stability and integrity, since displacement of Mg^{2+} by polymyxin makes the outer membrane permeable to periplasmic proteins and lysozyme (Warren *et al.*, 1957; Cerny and Teuber, 1971). Chelators like ethylenediamine tetraacetate (EDTA) are able to remove these Mg^{2+} ions, resulting in similar loss of outer membrane integrity. However, it is uncertain if the bactericidal or bacteriolytic activity of EDTA (Brown and Melling, 1969; Leduc *et al.*, 1982) is directly related either to outer or to cytoplasmic membrane damage.

E. Detergent Action

A variety of compounds, under the general category of surface-active agents or surfactants, have been shown to act as detergents in that, at moderate to high concentrations, they can solubilize biological membranes. Surfactants, which include a number of the molecules mentioned above, are amphiphilic in that they contain both hydrophobic and hydrophilic groups within the same molecule. They are classified into three groups according to charge: (1) anionic surfactants like sodium dodecyl sulfate (SDS), sodium deoxycholate (DOC), fatty acids, and phenolic antiseptics; (2) cationic surfactants like the quarternary ammonium anti-

septics and polymyxin B; and (3) nonionic surfactants like Triton X-100 and Tween 80. Although all of these surfactants can solubilize lipid bilayer membranes, many of them are thought to have modes of action separate from detergent solubilization (see above and Cornett and Shockman, 1978). However, the powerful anionic detergents SDS and DOC may well directly cause cytoplasmic membrane dissolution (Hotchkiss, 1946).

F. Effects on Membrane Functions

Essentially all of the agents resulting in either ion leakage, disruption, or detergent effects on cytoplasmic membranes also affect membrane functions as assayed by respiration activity (rate of O_2 consumption), (Ca^{2+},Mg^{2+})-ATPase activity, or transport of a variety of substrates (e.g., Teuber, 1974; Rosenthal *et al.*, 1977). In some cases, based on the kinetics of inhibition, it has been suggested that these are secondary effects resulting from the primary lesion (which is usually thought to be disruption of the protonmotive force, see above). However, for the antibiotic pamamycin, a direct inhibition of inorganic phosphate uptake in *Staphylococcus aureus* has been postulated as the likely primary target (Chou and Pogell, 1981).

G. Antibiotics, Thought to Have Other Primary Targets, Which Are Membrane Active

Many antibiotics have unusual chemical structures that differ greatly from normal cellular substrates. For this reason, it seems unlikely that they utilize cytoplasmic membrane substrate-transport systems in order to enter the cell. The passage of large antibiotic molecules across the cytoplasmic membrane may well be the cause of a variety of membrane-related phenomena that in turn may contribute to the action of the antibiotics on cells. For example, tetracyclines are thought to act primarily on protein synthesis but display a variety of side effects at high concentrations, including leakage of pools of nucleotides, amino acids, and sugars and inhibition of DNA replication (Pato, 1977). These side effects are thought to be related to an alteration by tetracycline of the cytoplasmic membrane. Novobiocin, which apparently acts primarily on the DNA gyrase B subunit to inhibit DNA replication, is also known to cause excretion of nucleotides and β-galactosidase (Brock and Brock, 1959). Chloramphenicol, which at high concentrations has as its primary target protein synthesis, can cause secretion of cell proteins, including β-galac-

tosidase, as well as nucleotides into the medium, with eventual bacteriolysis (Gupta, 1975). Many of these effects may be related to divalent cation supply in that high Mg^{2+} concentrations antagonize (i.e., prevent) the membrane effects of chloramphenicol (Gupta, 1975) and novobiocin (Brock, 1962) while tetracycline is a divalent cation chelator (Pato, 1977). Thus, it is tempting to hypothesize that these membrane-disruption events involve interaction of the antibiotics with Mg^{2+} binding sites (or Mg^{2+}-requiring processes) during their passage across the cytoplasmic membrane.

The primary mode of action of streptomycin and related aminoglycoside antibiotics is unclear, as summarized by Hancock (1981). A considerable number of membrane-related phenomena occur as a result of aminoglycoside action on cells, including K^+ and putrescine efflux as well as excretion of adenine nucleotides and β-galactosidase. Of these events, only K^+ efflux occurs prior to the lethal event (Dubin *et al.*, 1963; Hancock, 1981). In addition, aminoglycosides are known to cause disruption of the outer membrane permeability barrier in *P. aeruginosa* (Hancock *et al.*, 1981). While protein synthesis inhibition is certainly mediated by aminoglycoside antibiotics, it seems unlikely that this is itself the cause of the bactericidal action of aminoglycosides, and action on membranes may well be important (Hancock, 1981). Like the other antibiotics described in this section, aminoglycosides are strongly antagonized by Mg^{2+}.

IV. MECHANISMS OF RESISTANCE

A. The Outer Membrane as a Barrier

The outer membrane of gram-negative bacteria constitutes a permeability barrier to many antibacterial compounds. Hydrophilic compounds can pass across the outer membrane via the water-filled channels of proteins called porins (Nikaido and Nakae, 1979). The channel area of individual porin pores, and therefore the exclusion limit of porins and of outer membranes, varies from organism to organism [for example, *E. coli* porins allow the passage of trisaccharides or tetrapeptides whereas the *P. aeruginosa* porin excludes only those saccharides of molecular weight greater than about 6000 (Hancock and Nikaido, 1978; Nikaido and Nakae, 1979)]. Those outer membranes with smaller exclusion limits (e.g., *E. coli* and *Salmonella*) will exclude large antibacterial agents like polymyxins, gramicidin S, gramicidin A, and valinomycin and other ionophores (all 1200–1500 daltons) on the basis of size. Even in the case of *P. aeruginosa,*

which has a porin with a 60% larger channel diameter (Benz and Hancock, 1981), the large size and rigid cyclic structures of most of the above antibacterial agents, as well as the low activity of the *P. aeruginosa* outer membrane porin [only about 100–300 porins/cell form functional channels (Benz and Hancock, 1981; Angus *et al.*, 1982; Nicas and Hancock, 1983a)], would conspire to result in a very low rate of uptake via the hydophilic (porin-mediated) pathway. Since porin channels are filled with water and have charged amino acid residues at the mouth of the channel (Benz *et al.*, 1982), the movement of even small hydrophobic compounds through the channels will be severely restricted, as demonstrated experimentally by Nikaido (1976).

In addition to the porin-mediated, hydrophilic uptake pathway, two other mechanisms for antibiotic uptake across the outer membrane have been proposed, the hydrophobic pathway (Nikaido, 1976) and the "self-promoted" pathway (Hancock *et al.*, 1981). Nikaido (1976) has demonstrated in *Salmonella typhimurium* that the hydrophobic uptake pathway, which can broadly be considered as the dissolving of hydrophobic compounds into the membrane interior, is relatively unimportant in wild-type *S. typhimurium* or *E. coli* strains. Symptomatic of the lack of such a pathway is cellular resistance to hydrophobic antibacterials like actinomycin D, phenol, and crystal violet (Nikaido, 1976), as well as to detergents and bile salts (Nikaido and Nakae, 1979). Since this is a common property of wild-type gram-negative bacteria, with the possible exception of *Neisseria* (Maness and Sparling, 1973), it would seem that the hydrophobic uptake pathway is very inefficient in gram-negative bacteria in contrast to some but not all gram-positive bacteria. Even in deep rough (Re) mutants of *Salmonella*, which Nikaido (1976) showed to have a hydrophobic uptake pathway, the pathway can apparently be blocked by addition of divalent cations (Stan-Lotter *et al.*, 1979). This and other data suggest that the combined effects of divalent cation bridging of LPS molecules and high surface negative charge may be responsible for the absence of a hydrophobic uptake pathway in most gram-negative bacteria.

The self-promoted pathway has been postulated for polycationic antibiotics, like polymyxins and aminoglycosides, in *P. aeruginosa* (Hancock *et al.*, 1981). It involves the displacement of divalent cations from LPS by these polycations (Nicas and Hancock, 1983b), thus destroying the LPS cross-bridging and destabilizing the outer membrane. Since this can result in enhancement of uptake of lysozyme and β-lactams across the outer membrane (Hancock *et al.*, 1981), we have proposed that such interactions promote the uptake of the interacting polycationic antibiotic itself. As further evidence of self-promoted uptake, EDTA, a divalent cation chelator that removes Mg^{2+} from outer membrane sites, causes similar

enhancement of uptake of lysozyme and β-lactams as well as enhanced killing by the polycationic antibiotics (Sykes and Morris, 1975). Furthermore, a single point mutant of *P. aeruginosa* (Nicas and Hancock, 1980) renders the cell resistant not only to polycationic antibiotics but also to EDTA, whereas external Mg^{2+} antagonizes both classes of agents (Newton, 1954; Zimelis and Jackson, 1973). Although the self-promoted pathway has not been demonstrated in other bacteria, the similarity of polymyxin and EDTA effects on many bacteria, including *P. aeruginosa*, suggest it may well be a common pathway of uptake. A number of the membrane-active agents effective against gram-negative bacteria are cationic, including the quaternary ammonium disinfectants, chlorhexidine and other diguanidine compounds, gramicidin S, and tyrocidin as well as the polymyxins.

Thus, although the outer membrane has three potential pathways that can be utilized by antibacterial compounds, these pathways often cannot be utilized effectively by membrane-active compounds. The main exception seems to be the self-promoted pathway which can be utilized by polymyxins. The same surface features that prevent the existence of a hydrophobic uptake pathway in gram-negative bacteria also apparently result in the resistance of outer membranes, and consequently of cells, to detergents and other membrane-active agents (Nikaido and Nakae, 1979).

B. Detergents, Phenolic Compounds, and Cationic Antiseptics

In bacteria naturally resistant to detergents, phenolic compounds, and cationic antiseptics, insensitivity is generally due to failure of these substances to reach their targets in the cytoplasmic membrane; for example, some acid-fast gram-positive bacteria are relatively insensitive to a number of antiseptics, notably quaternary ammonium compounds, and the basis for this insensitivity is likely their relatively impermeable, waxy cell walls. Bacterial spores are also relatively insensitive, presumably due to lack of penetration of the antiseptics.

A number of gram-negative species, notably *P. aeruginosa*, other *Pseudomonas* spp., and *Proteus vulgaris*, are also characteristically resistant to cationic antiseptics. It appears that the basis for their resistance is the relative impermeability of the outer membrane of these organisms to such compounds, as suggested by studies in which disruption of the outer membrane by EDTA has been shown to increase susceptibility (MacGregor and Elliker, 1958; Haque and Russell, 1974).

Acquired resistance to cationic antiseptics has been demonstrated for several gram-negative species, including *Serratia marcecens*, *E. coli*, and

Klebsiella aerogenes as well as for *Pseudomonas* and *Proteus* spp. (Chaplin, 1952; Brown and Wood, 1972). Although the mechanism for this increase in resistance is not clearly established, it may involve alterations of the lipid composition of the cell envelope. In some cases, cross-resistance occurs between polymyxins and quaternary ammonium compounds (Anderes *et al.*, 1971; Brown and Tomlinson, 1979), and a mechanism of resistance involving decreased outer membrane permeation due to reduction in the number of surface binding sites has been suggested by Brown and co-workers (Brown and Wood, 1972; Brown, 1975). The acquisition of resistance of *P. aeruginosa* to chlorinated phenolic antiseptics under a variety of growth conditions has been attributed to an alteration in the LPS or phospholipid composition of the outer membrane that results in reduced outer membrane permeability (Gilbert and Brown, 1978).

An alternate mechanism of resistance to antiseptics is decomposition. Some instances of *Pseudomonas* spp. and other bacteria capable of degrading phenolic disinfectants such as methyl *p*-hydroxybenzoate, a preservative once commonly used in eye drops, have occasionally been documented (Hugo, 1967).

C. Polymyxins and Octapeptins

1. Intrinsic Resistance

Unlike the majority of membrane-active agents, polymyxins and octapeptins are more active against gram-negative than gram-positive bacteria. The natural resistance of many gram-positive bacteria and some gram-negative bacteria appears to be due to characteristics of the cell wall that prevent the penetration of polymyxin to the cytoplasmic membrane rather than to differences at the level of the cytoplasmic membrane itself. When the cell wall of resistant species is removed, for example by lysozyme in the case of gram-positive bacteria (Galizzi *et al.*, 1975) or in L forms of the naturally resistant species, *Proteus mirabilis* (Teuber, 1969), these organisms became polymyxin susceptible. Studies reviewed by Newton (1956) indicated that free cell envelopes of susceptible strains of both gram-positive and gram-negative bacteria were capable of adsorbing much more polymyxin than cell envelopes of resistant strains.

2. Polymyxin Antagonists

In addition to the acquired resistance described in the next section, the apparent susceptibility of gram-negative bacteria, especially *P. aeruginosa*, is profoundly influenced by the presence of divalent cations. This was first demonstrated by Newton (1953) who showed that Mg^{2+}, Ca^{2+},

and other divalent cations strongly inhibited the lytic activity of polymyxin B. He suggested that this inhibition was due to competition by divalent cations with polymyxin for membrane binding sites, which he postulated to be polyphosphate in nature. Subsequent studies have supported the conclusion that competition for the divalent cation binding site in the core region of LPS is involved in polymyxin activity (Brown, 1975; Nicas and Hancock, 1980). Schindler and Osborn (1979) have demonstrated high affinity binding sites for polymyxin B and for Mg^{2+} and Ca^{2+} in *S. typhimurium* LPS. The ability of polymyxin to compete with Mg^{2+} for membrane sites was demonstrated in *P. aeruginosa* by Nicas and Hancock (1983b) who were able to show displacement of 10% of cell envelope Mg^{2+} by treatment of whole cells with polymyxin. Furthermore, a variety of assays in both *P. aeruginosa* and enteric organisms have suggested that polymyxin disruption of outer membranes is strongly antagonized by divalent cations (Newton, 1954; Storm *et al.*, 1977; Hancock *et al.*, 1981).

Polymyxin activity is also inhibited by phospholipids, soaps, and other phosphatides. These agents act by binding polymyxins.

3. Mutational Resistance

Resistance to polymyxin B may be acquired either as a result of mutation or as unstable resistance brought about by specific growth conditions. In *P. aeruginosa*, Brown and Melling (1969) showed that growth in low concentrations of Mg^{2+} results in resistance to both polymyxin and EDTA. We (Nicas and Hancock, 1980), have isolated stable polymyxin-resistant mutants that when grown in Mg^{2+}-sufficient medium have the same properties as wild-type cells grown in Mg^{2+}-deficient medium. Both mutants and cells grown in low [Mg^{2+}] are resistant to EDTA as well as to polymyxins and show increased resistance to aminoglycosides when tested in a common assay medium (Hancock *et al.*, 1981). This resistance is correlated with greatly enhanced production of a major outer membrane protein H1, which is increased up to 24-fold in resistant cells (Nicas and Hancock, 1980).

This increase in protein H1 levels correlates with a decrease in divalent cation levels of the outer membrane. No measurable changes in the amount of LPS phosphate or in total membrane permeability (Nicas and Hancock, 1983a,b) and no major changes in phospholipid or fatty acid content (Gilleland and Conrad, 1982; R. A. Moore, L. Chan, and R. E. W. Hancock, in preparation) could be detected in the resistant cells. The mechanism of resistance proposed is that protein H1 acts by replacing divalent cations at a site on the LPS which can otherwise be attacked by

the polycationic antibiotics or by EDTA. This mechanism of resistance has not been found in other organisms, although we have observed protein H1 induction when *Pseudomonas putida* is grown under conditions of Mg^{2+} deficiency (T. Nicas, Ph.D. Thesis, University of British Columbia, 1982).

Since the model of polymyxin interaction with the outer membrane involves binding to LPS, it might be anticipated that LPS alterations which reduce available binding sites could provide an additional mechanism of resistance. However, the number of specific studies detailing LPS alterations in polymyxin-resistant strains is small. This owes itself, in part, to the difficulty of analyzing LPS structure, which is extremely complex (Luderitz *et al.*, 1982) and often inadequately understood. Even when a detailed study of LPS composition is performed and several indicators of LPS alterations obtained, e.g., in the study of an antibiotic (including polymyxin) supersusceptible mutant of *P. aeruginosa* (Kropinski *et al.*, 1982), the exact chemical nature of the mutation in LPS can evade definition by current technology. A significant problem in studies of polymyxin resistance is that the polymyxin interaction site is in the KDO lipid A portion of the LPS (Schindler and Osborn, 1979) that has been only 80% or less chemically defined.

LPS alterations affecting polymyxin resistance have been demonstrated in *S. typhimurium*. Vaara, Makela, and co-workers isolated a class of mutants (*pmrA*) that are polymyxin resistant and have LPS with reduced affinity for polymyxin (Vaara *et al.*, 1979; Vaara, 1981a). These mutants have an altered lipid A structure in which the ester-linked phosphate group is more highly substituted with 4-amino-4-deoxy-L-arabinose (60–70% substitution compared with 10–15% in wild-type). The mutants are also more resistant to the membrane-damaging effects of other cationic agents (e.g., polylysine and benzalkonium chloride) and EDTA (Vaara, 1981b).

As the activity of polmyxin on the cytoplasmic membrane appears to require binding to negatively charged phospholipids, several workers have investigated the possibility that lipid alterations could result in resistance. Although this is an attractive concept which has received much attention, few well-defined examples of this form of resistance have been found. One such mechanism of resistance has been shown in *Pseudomonas fluorescens,* where growth under phosphate-limiting conditions reduces the amount of membrane phospholipid and results in the synthesis of a novel cationic lipid, ornithine amine lipid (Dorner and Teuber, 1977). Alternatively, Brown (1975) has suggested that *P. aeruginosa* grown in low phosphate medium shows polymyxin resistance by virture of LPS alterations, since phosphate is a major component of *P. aeru-*

ginosa LPS and a potential polymyxin binding site. In each of the above cases, the alternative mechanism was not examined.

Decreased levels of wall phospholipid were also found in a stable, resistant mutant of *P. aeruginosa* (Brown and Wood, 1972). However, this strain also had reduced wall Mg^{2+} and was not characterized with respect to the outer membrane. Relatively low phospholipid content was also noted in resistant strains of *Klebsiella aerogenes* and *Proteus vulgaris* (Brown and Woods, 1972). In contrast, a correlation between lipid composition and polymyxin resistance could not be found for the polymyxin-sensitive and -resistant strains of *Proteus mirabilis* examined by Sud and Feingold (1970).

4. Adaptive Resistance

An interesting and unusual form of resistance is seen *P. aeruginosa* trained to grow on very high levels of polymyxin B (Brown and Watkins, 1970; Gilleland and Murray, 1976). This resistance is unstable and is lost when cells are grown on polymyxin-free medium. Extensive studies by Gilleland and co-workers have shown that the polymyxin-grown strains have a number of alterations including changes in lipid composition, reduced levels of Mg^{2+} and Ca^{2+}, reduction in major outer membrane proteins, and altered envelope morphology when examined by electron microscopy (Brown and Watkins, 1970; Gilleland and Murray, 1976; Gilleland and Lyle, 1974; Gilleland and Conrad, 1980). The authors suggest that this form of adaptive resistance is due to reduced permeability of the outer membranes to polymyxin while the cytoplasmic membrane remains sensitive (Gilleland and Farley, 1982). The change in permeability does not appear to be a generalized decrease since polymyxin-grown cells are considerably more susceptible to a number of other antibiotics, including most β-lactams, but more resistant to aminoglycosides.

Even stable, polymyxin-resistant, and protein H1-overproducing mutants of *P. aeruginosa*, when grown on low levels of polymyxin, demonstrate adaptive alterations in lipid content and outer membrane protein profiles (Gilleland and Conrad, 1982). This suggests that the mechanism of adaptation to polymyxin is distinct from the mechanism of resistance due to protein H1 overproduction reported by us (Nicas and Hancock, 1980, 1983b). In agreement with this, cells grown on Mg^{2+}-deficient medium [giving rise to an increase in protein H1 and in polymyxin resistance (Nicas and Hancock, 1980)] have different outer membrane freeze fracture profiles from polymyxin-adapted strains (Gilleland and Murray, 1976), and polymyxin-adapted strains do not show increases in protein H1 levels (Gilleland and Lyle, 1979; Gilleland and Conrad, 1982). These two

types of resistance probably represent alternative mechanisms of blocking the uptake of polymyxin across the outer membrane of *P. aeruginosa*. An apparently related form of resistance occurs in several species of *Proteus*, where growth on low levels of polymyxin leads to unstable resistance to high levels of the antibiotic (Shimizu *et al.*, 1977). The "coccarde" phenomena (growth at the edges of a polymyxin disk) similarly results in resistance of *Serratia marcescens* to high levels of polymyxin (Traub, 1982). It can be freely reversed by growth in the absence of polymyxin or by coaddition of a negatively charged detergent sodium deoxycholate, leading to the hypothesis that it involves the cationic detergent nature of polymyxins coating the cell surface and repelling other molecules of polymyxin (Traub, 1982).

Changes in fatty acid composition of readily extracted lipids have been seen in *P. aeruginosa* with enhanced polymyxin susceptibility due to growth on branched chain amino acids as the sole carbon source (Conrad *et al.*, 1979). Outer membrane protein patterns and LPS contents did not vary with the level of resistance in these studies (Gilleland and Conrad, 1980). An interesting form of adaptive resistance to polymyxin is provided by *P. aeruginosa* grown in hyperbaric (2 atm) oxygen (Kenward *et al.*, 1980), which gives rise to colonies 4- to 10-fold more resistant to polymyxin and cross-resistant to tetracycline and phenoxyethanol. Two colony types were noted: stunted rough colonies suggestive of LPS alterations and large mucoid colonies which had slight lipid alterations. The resistance was reversible upon incubation in normal air for 24 h.

It is thus clear that there are a variety of mechanisms for acquisition of polymyxin resistance as would be expected for an antibiotic that interacts with both outer and inner membrane components to exert its lethal effects. The common factor in most forms of resistance may be the reduction in the amount or availability of negatively charged lipidic components, either phospholipid or LPS, with which the antibiotic may interact.

D. Chelators of Divalent Cations

EDTA causes increased outer membrane permeability in many gram-negative bacteria (Leive, 1974). *Pseudomonas aeruginosa* and some other *Pseudomonas* spp. are exceptionally susceptible to EDTA and can be rapidly killed by EDTA-Tris treatment. Wilkinson (1968) showed that high levels of cell envelope phosphate and Mg^{2+} were characteristic of EDTA-sensitive pseudomonads but not of resistant *Pseudomonas* species or enteric gram negatives. It would appear that the divalent cation binding sites attacked by EDTA are either more numerous or more critical to

membrane stability in sensitive *Pseudomonas* species. Much evidence indicates that the site of EDTA activity on the outer membrane of *P. aeruginosa* is the same (LPS) site attacked by polymyxins (Brown and Melling, 1969; Brown, 1975) since cross-resistance to both of these agents is seen in cells grown in low [Mg^{2+}] and in polymyxin-resistant mutants. Nicas and Hancock (1980 and 1983b) have described one mechanism of cross-resistance to EDTA and polymyxin (see Section 4 above). *Pseudomonas aeruginosa* grown in the presence of Ca^{2+} is sensitive to the Ca^{2+}-specific chelator ethyleneglycol bis(β-aminoethyl ether) *N,N'*-tetraacetate (EGTA) but is resistant when grown in the absence of Ca^{2+}. It thus appears that Ca^{2+} can substitute for Mg^{2+} in the sites involved in EDTA and polymyxin activity (Boggis *et al.*, 1979; Nicas and Hancock, 1983b).

Although *Salmonella* is more resistant than *P. aeruginosa* to killing by EDTA, polymyxin-resistant mutants (*pmrA*) of *S. typhimurium* (Vaara, 1981b) described above are also resistant to the outer membrane damaging effects of EDTA. For example, they are resistant to killing by EDTA-lysozyme, EDTA-bacitracin, and EDTA-deoxycholate as well as to EDTA-promoted release of LPS. This suggests that a common outer membrane site for polymyxin and EDTA action also occurs in other bacteria.

E. Other Cyclic Peptides

Cyclic peptide antibiotics, such as the tyrocidins and gramicidin S, are produced by sporulating bacteria and are thought to have functions related to the sporulation process. The mechanisms by which these bacteria avoid being killed by their own antibiotics are not understood. Gram-negative bacteria are generally quite resistant to these antibiotics due to outer membrane impermeability (see Section IV,A).

F. Bacteriocins

There are two general mechanisms by which a bacterial strain may lose its susceptibility to membrane-active bacteriocins: loss of the surface receptors for the bacteriocin, usually termed "resistance," and alteration of a component involved in the subsequent uptake and action of the bacteriocin (including the energy coupling systems of the cytoplasmic membrane), usually termed "tolerance" (Reeves, 1972; Konisky, 1979). The generally narrow species-specificity of bacteriocins is probably due to their requirement for specific outer membrane protein or LPS receptors. Many bacteriocin-resistant mutants lacking specific receptors have been

isolated, and such mutants have proven useful in the study of outer membrane proteins. Tolerant mutants can show pleiotropic effects on membrane functions related to energy transduction. For example, temperature-dependent colicin K-tolerant mutants isolated by Plate (1976) were unable to grow on succinate and showed reduced proline transport at temperatures where colicin K tolerance was expressed.

Hong and co-workers (1979) have demonstrated that tolerance to colicins K and E1 results from a mutation in the *ecf* gene, a locus essential for coupling of active transport and oxidative phosphorylation. A mechanism of tolerance to colicins Ia and B also related to membrane energy coupling function was described by Konisky (1975), who isolated tolerant mutants that had reduced rates of respiration on succinate and low levels of succinate dehydrogenase but normal respiration rates on glucose and elevated levels of NADH oxidase. Alternatively, tolerance can also be caused by outer membrane alterations (Davies and Reeves, 1975), presumably reflecting the role of certain outer membrane components in bacteriocin uptake.

Bacteria capable of producing a given bacteriocin are generally not affected by that bacteriocin. For colicins E3 and E1 this has shown to be due to specific "immunity" proteins which complex with the bacteriocin and block its activity (Luria, 1973).

G. Other Agents

1. Uncouplers

Mutants resistant to uncouplers have been sought for their potential usefulness in the study of energy coupling. These include mutants that are altered in various parts of the cytoplasmic membrane-associated, proton-translocating ATPase complex. Examples include mutants of *Bacillus megaterium* and *E. coli,* which are resistant to carbonyl cyanide *m*-chlorophenylhydrazone, 2,4-dinitrophenol, and pentachlorophenol and have lost ATPase hydrolytic activity (Decker and Lang, 1977, 1978; Ito and Onishi, 1982), and mutants of *E. coli* and *Streptococcus faecalis* altered in the membrane bound portion of ATPase, Fo (*uncE* gene product), and resistant to *N,N'*-dicyclohexylcarbodiimide (Filligame, 1975; Leimgruber *et al.,* 1981).

2. Cyanide

Resistance to hydrogen cyanide, a potent inhibitor of respiration, has been described in a number of bacterial species, including two species of

bacteria, *P. aeruginosa* and *Chromobacteria violaceum*, capable of producing toxic levels of cyanide during growth (Knowles, 1976; Kralik and Castris, 1979). Mechanisms of cyanide resistance have been reviewed by Knowles (1976). Cyanide resistance may occur as a result of metabolic breakdown of cyanide or as a result of switching from primarily cyanide-sensitive respiration to respiration using relatively cyanide-resistant cytochrome oxidases. In experiments with *Achromobacter*, for example, it has been shown that cytochrome *d* oxidase is KCN insensitive, and cells growing in the presence of KCN had 10 times the levels of this cytochrome (Arima and Okai, 1965).

V. METHODS FOR OVERCOMING RESISTANCE

A. Ethylenediamine Tetraacetate (EDTA) and Other Chelators

Since the work of Leive (1974), it has been clear that EDTA, a divalent cation chelator, permeabilizes outer membranes. As discussed above, in the absence of EDTA, outer membranes provide a significant barrier to hydrophobic and large hydrophilic compounds and at the same time significantly reduce the rate of permeation of smaller hydrophilic antibiotics into the cell (Nikaido and Nakae, 1979; Angus *et al.*, 1982). EDTA probably acts on outer membranes by removing the divalent cations which cross-bridge adjacent LPS molecules. This would then cause charge repulsion between adjacent LPS molecules with consequent disruption and loss of barrier function of the outer membrane. Other chelators cause similar effects (Roberts *et al.*, 1970), although there is some specificity since EGTA, a calcium-specific chelator, will only permeabilize the outer membranes of cells grown on Ca^{2+} as sole divalent cation but not of Mg^{2+}-grown cells (Nicas and Hancock, 1983a).

EDTA has been reported to potentiate the action of the membrane-active agents, quaternary ammonium disinfectants (MacGregor and Elliker, 1958; Haque and Russell, 1974), chlorhexidine diacetate (Brown and Richards, 1965), chloroxylenol (Gray and Wilkinson, 1965), polymyxin B, and ionophores (Brown and Richards, 1965), as well as a number of other agents that cause cell membrane effects including aminoglycosides, tetracycline, and chloramphenicol (Weisser *et al.*, 1968; Davis and Iannetta, 1972). In a practical sense, EDTA does not seem to be the ideal chelator to use since high doses might cause hypomagnesemia and hypocalcemia. Despite this, clinical trials with EDTA as a potentiator of antibiotic action have been quite promising (Wilson, 1970). Alternative chelating agents

which also show promise are triethylamine dichloride (Light and Riggs, 1978) and ascorbic acid (Rawal, *et al.*, 1974), although these have not been tested for synergy with membrane-active agents.

B. Synergistic Combinations

It has been proposed that polymyxins act at a site similar to divalent cation chelators like EDTA, displacing rather than removing Mg^{2+} from the LPS and consequently permeabilizing the outer membrane (Brown, 1975; Hancock, *et al.*, 1981). Thus, it is perhaps not surprising that synergism between polymyxins and either tetracycline (Makrigiannis and Gaca, 1971), carbenicillin (Smith *et al.*, 1969), complement + lysozyme, (Fierer and Finley, 1979), normal human serum (Sud and Feingold, 1975), sulfonamides (Greenfield and Feingold, 1970), bacitracin (Rosenthal and Storm, 1977), rifampicin (Rosenthal and Storm, 1977), deoxycholate (Sud and Feingold, 1972), the nonionic surfactant polysorbate 80 (Brown *et al.*, 1979), or the phenolic disinfectant chlorhexidine (Al-Najjar and Quesnel, 1979) has been observed in gram-negative bacteria. However, the basis of the observed synergism between anionic surfactants and sulfydral reagents (Bernheim, 1978) and between di- or tricarboxylic acids and sodium dodecyl sulfate (Adair *et al.*, 1979) is yet to be explained, although plausibly it could be related to the ability of sulfydral reagents and tricarboxylic acids to act as chelators of divalent cations (see Section V.A above for mechanism of chelator action).

C. Polymyxin Resistance

Acquired resistance to polymyxin B in *P. aeruginosa* has been shown to be reversed by growth in the absence of polymyxin (Gilleland and Murray, 1976) or by appropriate medium supplementation (Brown and Melling, 1969; Nicas and Hancock, 1983b) depending on the mechanism of resistance. Acquired polymyxin resistance in *Serratia marcescens* (coccarde phenomenon) can be simply reversed by sodium deoxycholate (Traub, 1982).

VI. CONCLUSIONS

In general, membrane-active agents are either hydrophobic agents like ionophores, etc., that are largely excluded by the gram-negative outer membrane or have specific structures like polymyxin B, the action of

which depends on the presence of susceptible sites on the cell surface. It is therefore hardly surprising that such compounds have limited therapeutic usefulness other than for topical infections or as general disinfectants. The problem of obtaining a membrane-active, antibacterial agent that is effective against most bacterial membranes but largely inactive against eukaryotic membranes may well be unsolvable due to the similarities between lipid bilayers from prokaryotes and eukaryotes.

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