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Molecular Basis of Bacterial Outer Membrane Permeability

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INTRODUCTION

Bacteria produce cell walls with the exception of mycoplasmas, and the cell wall component common to all eubacteria is the murein, or the peptidoglycan, which contributes mechanical rigidity. All gram-negative bacteria contain an additional layer in the cell wall structure, i.e., the outer membrane, which is located outside the peptidoglycan layer and shows up as a trilaminar structure on the electron micrographs of thin sections of these bacteria (84).

It is becoming increasingly clear that the outer membrane is very important in the physiology of gram-negative bacteria in making them resistant to host defense factors such as lysozyme, β-lysin, and various leukocyte proteins, which are very toxic to gram-positive bacteria (57, 230, 242). In enteric gram-negative bacteria, which live in the intestinal tract of animals, the outer membrane has developed into a very effective barrier, giving protection to cells from the detergent action of bile salts and degradation by digestive enzymes (207). At the same time the outer membrane of enteric and some other gram-negative bacteria acts as a strong permeability barrier to many antibiotics that are effective against other bacteria (e.g., macrolides, novobiocin, rifamycins, lincomycin, clindamycin, and fusidic acid; see reference 207). Even when the diffusion of antibiotic is merely slowed down by the presence of outer membrane, the bacteria can then inactivate the small amount of penetrating antibiotic rather than try to inactivate the almost infinite amount of antibiotic present in the medium, and thus very high levels of resistance are easily established in

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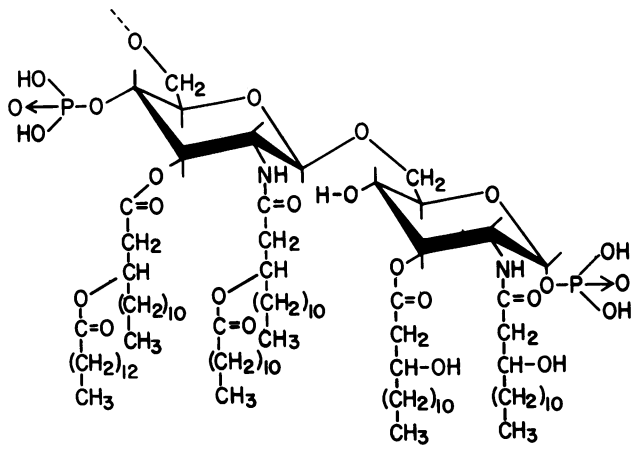

FIG. 1. Structure of lipid A from the LPS of *S. typhimurium*. The polysaccharide chain is linked to C-6 of the nonreducing glucosamine residue; its site of attachment is shown by the dotted line. Although the particular subspecies of lipid A analyzed here contained six fatty acid residues, other subspecies, possibly from other strains, may contain seven fatty acid residues (see, for example, reference 334). From reference 294 with permission of the American Society of Biological Chemists.

gram-negative bacteria (see, for example, reference 246). These facts are obviously related to the prevalence of gram-negative infections in the modern hospital environment. We emphasize here that all of these phenomena are related to the function of the outer membrane as an effective permeability barrier.

Another important function of the outer membrane is to endow the bacterial surface with strong hydrophilicity, which is important in evading phagocytosis, some complement resistance, and the capacity to avoid a specific immune attack by altering the surface antigen constitution (158). It should be noted that these surface functions as well as the construction of a highly impermeable membrane layer noted above involve lipopolysaccharide (LPS), a characteristic component of the outer membrane, and that these are probably the physiological functions of LPS for the bacterial cell.

The bacterial outer membrane has been an object of intensive research during the last decade, and a number of reviews (general—151, 201, 207; proteins—56, 220; transport functions—127, 202, 203, 206; H. Nikaido, Pharamocol. Ther., in press; genetics—162; and a book—117) have appeared. Because of this, we do not try to give an exhaustive account in this review, but rather to present a critical review on the organization of the outer membrane and its role in the modulation of permeability. We exclude the following areas, which are covered in the recent reviews cited: "cell surface" functions, especially in interaction with the environment, including host cells (8, 20, 62); interaction with colicins and phages (128, 151); and assembly of the outer membrane (171, 199, 218, 275, 276). Even in the areas covered, we had to be very selective in citing references to limit the length of this review, and we apologize to the authors of the articles we could not quote for this reason.

Because most extensive studies have been carried out with *Escherichia coli* and *Salmonella typhimurium*, we usually begin each section of this review by describing these results, sometimes without specifically mentioning the names of these species. This is followed by the description of results obtained with other bacteria, when such results are available and pertinent.

COMPONENTS

Phospholipids

Phospholipid composition of the outer membrane is usually very similar to that of the cytoplasmic membrane, with a slight but significant enrichment in phosphatidylethanolamine in *S. typhimurium* (219). Lugtenberg and Peters (152) have reported that the proportions of phosphatidylethanolamine in outer and inner membranes of *E. coli* are higher than in corresponding membranes of *S. typhimurium*; this is consistent with the observation (H. Nikaido, unpublished data) that it is more difficult to make liposomes from *E. coli* cell envelope lipids than from *S. typhimurium* envelope lipids, in view of the well-known difficulty of producing bilayer dispersions of phosphatidylethanolamine.

LPS

The structure and properties of LPS have been discussed extensively in various reviews (77, 148, 199) and in a series of monographs on LPS, the first of which, dealing with the chemistry (248), has just appeared. We note, however, that significant advances have been made recently on the structure of lipid A, the hydrophobic membrane-anchoring region of LPS (115, 237, 286, 294) (Fig. 1).

The structure of LPS from *S. typhimurium* and *E. coli* K-12 is shown in Fig. 2. There are several important points that are relevant to our discussion. (i) Unlike phospholipids, which have only two fatty acid chains connected to the backbone structure, LPS molecule has six or seven fatty acid chains linked to the glucosamine disaccharide backbone (Fig. 1). (ii) Unlike in phospholipids, all of the fatty acid chains in LPS are saturated, and some are 3-hydroxy fatty acids. Furthermore, some fatty acid residues are linked to the 3-hydroxy group of another fatty acid, producing the characteristic 3-acyl-oxy-acyl structure (Fig. 1). (iii) Many negatively charged groups exist on the backbone itself as well as on proximal sugar residues such as 2-keto-3-deoxyoctonic acid (more correctly, 3-deoxy-D-*manno*-octulosonic acid). Indeed, LPS was found to bind divalent cations quite strongly (261), and the nature of counter ions have a profound influence on the physical structure of LPS aggregates (76). (iv) Many mutants producing defective LPS molecules have been isolated (162); these defective LPS molecules define different regions in the complete LPS (Fig. 2). For example, *rfb* mutants produce Ra LPS containing the complete core but totally lacking the peripheral O polysaccharide, and *rfaC* mutants produce Re LPS (or more correctly, a glycolipid) lacking all of O polysaccharide and most of the core oligosaccharide, except the 2-keto-3-deoxyoctonic acid residues.

Polysaccharide Components Other Than LPS

Enterobacterial common antigen (ECA) is an acidic polysaccharide containing *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosaminuronic acid, and 4-acetamido-4,6-dideoxy-D-galactose (149) and is present in a significant amount in cells of *Enterobacteriaceae* (159, 166). ECA appears to be linked to a phospholipid "anchor" (133), which presumably attaches ECA to the outer membrane. In the so-called ECA-immunogenic strains, however, ECA is linked to the LPS core.

Many bacteria have, as an outermost surface structure, a capsular layer usually consisting of acidic polysaccharides. At least some of these polysaccharides also have lipid components (88, 217, 267).

Proteins

Nearly half of the mass of the outer membrane is protein (219). Most outer membrane proteins are thought to be located exclusively in the outer membrane, although some proteins are found in both the outer and cytoplasmic membranes. In contrast to earlier conclusions that outer membrane contained just a few protein species, the number of outer membrane proteins seems to be fairly large if a sufficiently sensitive method of detection is used. However, the protein pattern is usually dominated by a few, so-called "major" proteins. These include the porins and the OmpA protein in the approximately 35,000-dalton range and the murein lipoprotein with a much lower molecular weight. (The arrangement of these proteins in the outer membrane is schematically shown in Fig. 3.) In addition, when *E. coli* cells are grown in maltose, the phage lambda receptor protein or LamB protein becomes a major protein, and under iron starvation conditions the proteins involved in the uptake of the ferric chelator complexes become predominant protein species (167, 235).

Murein lipoprotein. Lipoprotein is a small (7,200-dalton) protein that exists in a large number of copies (7×10^5) per cell. About one-third of the population of this protein occurs in a form bound covalently to the peptidoglycan layer through the $-NH_2$ group of its C-terminal lysine, whereas the rest occur as free proteins (118). The N-terminal residue, cysteine, is modified in an unusual manner: its sulfhydryl group is substituted with a diglyceride, and its NH_2 group is substituted with a fatty acid residue through an amide linkage (29). The amino acid sequence of lipoprotein has been determined (29), and the polypeptide chain appears to exist mostly in α-helical form, as expected from the sequence (30). Cross-linking showed that lipoproteins tend to get cross-linked with each other (239), and thus oligomeric association is possible. There is no clear evidence indicating the exposure of lipoprotein on the cell surface, and in view of the strongly hydrophilic amino acid composition it seems probable that only its lipid portion penetrates into the outer membrane.

The mutants with deletions through the structural gene for the lipoprotein are quite viable, and therefore the protein is not essential for growth under laboratory conditions (109). These mutants are also normal in the diffusion of small, hydrophilic solutes (205). However, the cell wall structure in these mutants appears to be unstable, resulting in the release of outer membrane vesicles and periplasmic enzymes (109, 289). It therefore seems reasonable to assume that the main function of the protein is a structural one in that it stabilizes the architecture of the outer membrane-peptidoglycan complex by holding down the outer membrane to the surface of the peptidoglycan.

Recently, a series of minor lipoproteins which presumably share a similar lipid structure at their N termini have been discovered (114). The function of these proteins is unclear.

OmpA protein. The OmpA protein in *E. coli* has a monomer molecular weight similar to that of porins, but behaves very differently upon solubilization in sodium dodecyl sulfate (SDS). Thus, the mobility of the protein in SDS-polyacrylamide gel electrophoresis decreases significantly when the preparation is heated in SDS. This behavior, often called "heat modifiability" in earlier literature, is quite characteristic of this protein (192, 268), and major outer membrane proteins showing similar behavior have been reported in some other species, for example, protein II or opacity protein of *Neisseria gonorrhoeae* (102, 290) and the group III protein of *Brucella* spp. (323). Unlike *E. coli* porins, the OmpA protein does not form an SDS-resistant oligomer. Although OmpA protein can be cross-linked to another OmpA protein in the outer membrane (225), it is unlikely that the protein exists as a population of homogeneous oligomers, as indicated by an X-ray diffraction study (305). The OmpA protein seems to be often associated with lipoprotein (225).

The OmpA protein of *E. coli*, like the porins, is rich in β-sheet structure (192). It appears to span the thickness of the membrane, as it can be labeled by a nonpenetrating reagent in intact cells (122) and serves as a phage receptor (see reference 151), whereas at the same time it can be cross-linked to the underlying peptidoglycan layer (66). An *ompA* mutant showed reduced overall transport rates for amino acids (165), and two *ompA* mutants belonging to separate lines were recently found to be defective in peptide transport (J. Payne, personal communication). On the other hand, an *ompA* mutant of *S. typhimurium* showed an unaltered permeability to cephaloridine (211). Obviously more

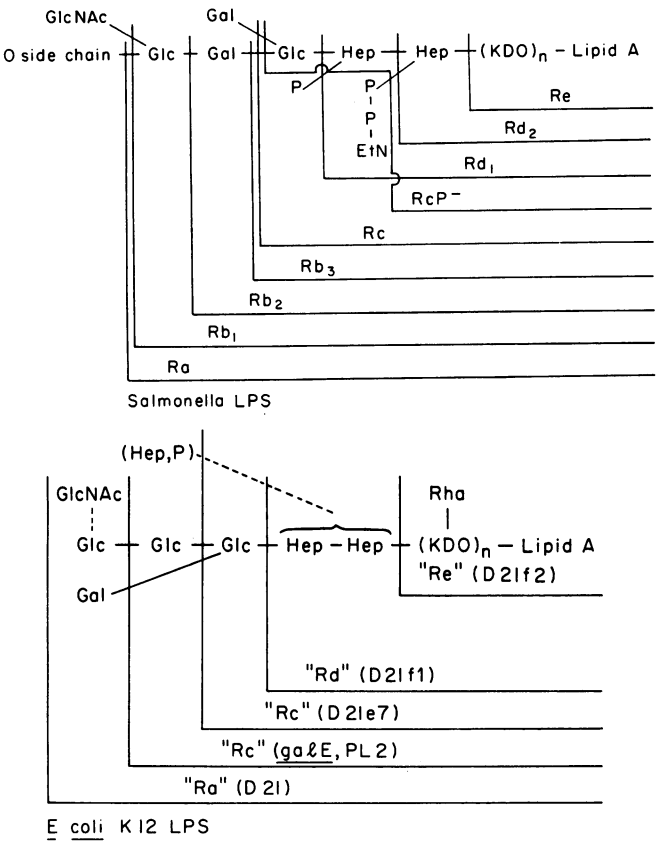

FIG. 2. Structure of LPS from *S. typhimurium* (top) and *E. coli* K-12 (bottom). Ra through Re refer to the chemotypes of the mutant LPS produced. It should be noted that the chemotypes of *E. coli* LPS do not correspond exactly to the series of Ra through Re, as many of them contain L-rhamnose (Rha) linked to 3-deoxy-D-*manno*-octulosonic acid (2-keto-3-deoxyoctonic acid [KDO]). With K-12, representative strains showing each phenotype are given in parentheses. The number of KDO residues, n, was generally thought to be 3, but some more recent work suggests 2. Broken lines indicate incomplete substitution. The salmonella LPS structure is based on reference 77; that of *E. coli* is based on references 232, and 233. Other abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; Glc, D-glucose; Gal, D-galactose; Hep, L-glycero-D-*manno*-heptose; EtN, ethanolamine; P, phosphate.

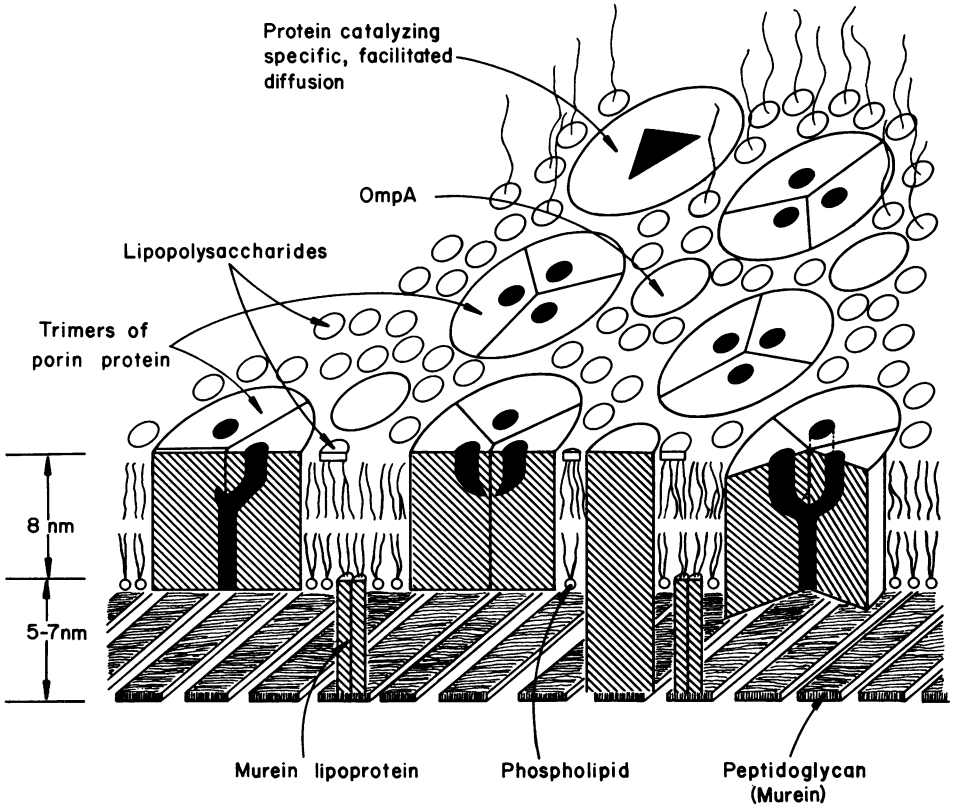

FIG. 3. Schematic model of the *E. coli* and *S. typhimurium* outer membrane. Note that some features (such as the length of the saccharide chain of LPS) are not drawn to scale. The specific channel is drawn as a monomer for simplicity; LamB protein is known to exist as a trimer, and the quaternary structure of the Tsx protein has not been established. Reprinted from reference 307a with permission of the publisher (Elsevier Science Publishers).

work is required in this area, but the formation of pores by the OmpA protein itself seems unlikely on the basis of the data on the porin-deficient mutants (see below). It is known that mutants lacking the OmpA protein produce unstable outer membrane and are defective in conjugation functions (164). The OmpA protein of *E. coli* has been sequenced (38). A striking finding is the Ala-Pro-Val-Val-Ala-Pro-Ala-Pro-Ala-Pro-Ala-Pro sequence at residues 176 to 187, a sequence resembling the "hinge" region of immunoglobulins. Indeed this exposed, protease-sensitive sequence appears to separate the protein into two large domains, the N-terminal domain inserted into the outer membrane and the C-terminal domain presumably exposed on the inner side of the membrane (38).

Porins. The proteins coded for by *ompF*, *ompC*, and *phoE* genes in *E. coli* K-12 are called porins because they produce relatively nonspecific pores or channels that allow the passage of small hydrophilic molecules across the outer membrane. In addition to the evidence on the reconstitution of purified porin into proteoliposomes (184, 185), the physiological importance of porin in allowing a diffusion of nutrients, antibiotics, or inhibitors across an outer membrane has been established by the use of porin-deficient mutants (13, 19, 154). Other strains of enteric bacteria sometimes produce additional porins. Examples include the OmpD porin of *S. typhimurium* LT2 (216), the prophage-coded protein 2 or Lc of *E. coli* (236, 269), and protein K, which is found among encapsulated strains of *E. coli* (224, 288, 331). Proteins with similar functions have been identified in several other bacteria. Their properties are described in more detail in the section, "Nonspecific Protein Channel: Porins."

Proteins involved in specific diffusion processes. The LamB protein of *E. coli* is involved in allowing the passage of maltose and maltodextrins through the outer membrane. Its properties are discussed in more detail below. Several other proteins are known to be involved in the transmembrane diffusion of specific groups of solutes. These include the Tsx protein or T6 receptor (involved in nucleoside transport), the TonA (FhuA) protein or T1,T5 receptor protein (involved in the uptake of ferrichrome), the FepA protein (involved in the uptake of Fe^{3+}-enterochelin), and the BtuB protein (involved in the uptake of vitamin B_{12}). They are described in the section, "Specific Diffusion Channels."

Other proteins. A few enzymes have been located in the outer membrane. They include phospholipase A_1 (213) and protease(s) (70, 156). A protein with a strong affinity of LPS (82) has been prepared from *Salmonella minnesota* by extraction with salt solutions. This is an interesting observation in view of the protein-LPS interaction in the outer membrane (see below). However, a major protein with identical mobility does not appear to have been reported in the analysis of cell envelopes performed in several laboratories, and the possibility that it is a proteolytic fragment of another protein cannot be excluded at present.

NONSPECIFIC DIFFUSION CHANNEL: PORINS

Properties of Porins

Outer membranes of gram-negative bacteria must allow the influx of nutrients and efflux of waste products. Indeed it was found that the outer membrane of enteric bacteria was

permeable to hydrophilic solutes of <600 daltons (54, 190). Nakae and Nikaido made a systematic search for the component of the outer membrane responsible for this permeability property by using an in vitro reconstitution approach. After negative results with LPS (189), some positive results were obtained with the crude protein mixture from the outer membranes (184), and purification of the protein by following the channel-forming activity led to the isolation of porin (185), which turned out to be identical to the protein earlier characterized by Rosenbusch (251) and called "matrix protein" because it was believed to be responsible for the presumed shape-determining function of the outer membrane. Since there is no evidence that porins contribute to the maintenance or determination of cell shape (272), the use of the term matrix protein should be discouraged. (More recently, the porins were claimed to be important in the "maintenance" of cell surface structure, because a porin-deficient mutant did not show an undulated surface morphology upon negative staining after treatment with a Tris buffer of high concentration [0.12 M] [214]. However, this is more likely to reflect the easier removal of LPS by the Tris cation [see below] in the mutant, and does not demonstrate any structural function for porin.)

Porins have been isolated and studied extensively in the laboratories of Schnaitman (11, 268, 269), Rosenbusch (79, 80, 251, 252), Henning (37, 267a), and Nakae (188, 303). In both *E. coli* and *S. typhimurium* multiple species of porin are found in a single strain. *E. coli* K-12 strains contain OmpF and OmpC porins when grown under normal culture conditions, and PhoE porin is added to these when cells are grown under phosphate starvation (9, 304). *S. typhimurium* LT2 cells grown in ordinary media contain three porins, OmpF, OmpC, and OmpD, earlier referred to as 35K, 36K, and 34K proteins, respectively (216).

Porins from both *E. coli* and *S. typhimurium* exist as undenatured trimers when extracted with SDS (188, 226, 251, 303). Circular dichroism and infrared spectroscopy showed that they were unusually rich in β-sheet structure and that there were no detectable α-helical segments (192, 251). The primary structure of the *E. coli* OmpF porin was determined in the laboratory of Henning (37), and the amino acid sequences of OmpF, OmpC, and PhoE proteins were deduced from the nucleotide sequences of respective genes (116, 172, 222). There is very strong homology among the three sequences (for comparison see reference 172). Secondary-structure prediction by the method of Chou and Fasman (39) or Garnier et al. (81) shows regions that are predicted to assume different conformations, but analysis of hydrophobic moment by the method of Eisenberg et al. (63) shows strong similarity among the three proteins even in these regions (H. Nikaido, unpublished data).

The sequences do not show any long stretches of hydrophobic amino acid residues. However, the secondary structure predictions show many 11 to 15-residue stretches which are predicted as β-sheets and contain only a few, if any, charged residues. Since β-sheet is a much more extended conformation than the α-helix and can cross the thickness of the membrane in 11 to 12 residues, it is tempting to imagine that the protein crosses the membrane many times by using these stretches of β-sheets. Infrared analysis has shown that many of the β-sheet structures of porin are oriented so that the backbone is roughly perpendicular to the surface of the membrane (80). Studies of the three-dimensional structure of porin, using monoclonal antibodies (P. Klebba and H. Nikaido, in preparation) and a covalent labeling approach (J.-M. Schlaeppi, S. Ichihara, and H. Nikaido, submitted for publication), are in progress, but the detailed structure should soon be available from X-ray crystallographic analysis currently in progress in the laboratory of R. Garavito and J. Rosenbusch (80).

E. coli porins tend to form two-dimensional hexagonal crystals when other proteins have been removed from the outer membrane by extraction with SDS (251). Electron microscopic analysis of these preparations with computer refinement has produced a wealth of significant information. It was found that each unit, presumably corresponding to a trimer, contained a triplet of holes or indentations that retained the negative stain (58, 282). (Triplets of pores have recently been seen also in unstained, frozen-hydrated specimens [C.-F. Chang, S. Mizushima, and R. M. Glaeser, Biophys. J., in press].) Furthermore, analysis of a tilted specimen showed that these three channels became fused in the middle of the membrane and exited on the other side of the membrane as a single central channel (59) (Fig. 3). These studies show clearly why the tight association of the three subunits is necessary for the production of a functional channel.

Porins have been identified in several other gram-negative organisms. These include *Serratia marcescens* and *Proteus vulgaris* (L. S. Zalman, Ph.D. thesis, University of California, Berkeley, 1982), *Pseudomonas aeruginosa* (94), *Aeromonas salmonicida* (51), *Chlamydia trachomatis* (14), *Rhodopseudomonas capsulata* (71), *Rhodopseudomonas sphaeroides* (329), *Paracoccus denitrificans* (L. S. Zalman and H. Nikaido, J. Bacteriol., in press), *N. gonorrhoeae* (60), and *Brucella* species (61). In the cases studied, they all seem to share the property of the enterobacterial porin in terms of the abundance of β-sheet structure (71, 329). However, the porins from *Paracoccus denitrificans* (Zalman and Nikaido, in press) and *Rhodopseudomonas* species (Z. Z. Yan et al., in preparation) appear to exist as dimers as judged on the basis of cross-linking studies. It is also interesting that the stability of porin oligomers spans a wide range depending on their source. At one end, the trimers from *E. coli* and *S. typhimurium* are quite stable and cannot be dissociated until the subunits are denatured by heating in SDS. At the other extreme, the porin from *P. aeruginosa* dissociates into monomers even when extracted with SDS at room temperature (94). The oligomer of *R. sphaeroides* porin seems to have an intermediate degree of stability and is stable at room temperature in SDS, yet is dissociated if EDTA is added (329).

The monomers of *E. coli* porin are inactive in forming channels, as they are denatured during the process of dissociation. The monomers of *P. aeruginosa* (339) and *R. sphaeroides* porin (329), however, show strong channel-forming activity in reconstitution assays. At present it is not clear whether the porins can form channels without aggregating into oligomers within the bilayers of the liposome.

E. coli porins, and probably also porins from other sources, are known to have a strong affinity toward LPS, as shown by the persistence of LPS in purified porin preparations. However, there is a wide variation in the reported extent of this contamination, some laboratories finding only 0.2 to 0.3 mol of LPS per trimer (74, 223) and another finding as high as 9 mol of LPS (259). The potential effect of the associated LPS on the function of porin is discussed in the next section.

The *E. coli* OmpF and OmpC porins act as receptors for phage TuIa and phages TuIb and Me1, respectively, but the isolated porins suspended in Triton X-100 in bicarbonate buffer, or complexed with phospholipids, did not inactivate these phages, and reconstitution of the receptor activity

required the addition of LPS (52, 318). More recently, Yu et al. (342) showed that lipid A did not substitute for LPS in the reconstitution of TuIb receptor and that LPS isolated from an Re mutant of *E. coli* was very poorly active in reconstitution. These results may indicate that OmpC porin must interact with the core region of LPS to undergo a conformational change, before it can act as a phage receptor. Alternatively, OmpC protein and LPS may constitute two independent parts of the receptor complex, both of which are essential for the successful inactivation of TuIb. A somewhat different system exists in the case of the receptor for phage T4; LPS containing nonreducing terminal glucose (Fig. 2) can act as receptors by itself, but LPS with different structures require the presence of OmpC protein to inactivate the phage (341).

We have already mentioned the tendency of *E. coli* porins to form ordered hexagonal arrays when other proteins are removed from the cell envelope by extraction with SDS (251). Such arrays can be formed in the absence of the peptidoglycan layer (282, 335), and LPS (or even lipid A or fatty acids) stimulate this process (335). This two-dimensional crystal was obviously useful in the study of the structure of porin, as mentioned earlier. However, it also led some people to believe that such a crystalline structure existed in intact cells. We do not think this is a valid idea. (i) In intact outer membranes, there are other transmembrane proteins, and obviously their presence will interfere with the growth of porin crystals. Furthermore, *E. coli* B, used by Rosenbusch (251), is unusual in containing only the OmpF porin. Other strains of enteric bacteria contain multiple species of porin, and this heterogeneity should also hinder the crystallization process. (ii) Extensive freeze-etching and -fracturing studies were carried out in several laboratories. Smit et al. (278) found no evidence of crystalline arrangement of protein "particles" in the outer membrane of *S. typhimurium*, and even with *E. coli* B evidence of regular arrangement could be found only in extremely rare, small areas (17). These results were confirmed recently by Verkleij et al. (cited in reference 151) under the conditions that should be most favorable for crystallization, i.e., by using a mutant of *E. coli* B missing the OmpA protein. (iii) The most decisive data are those of Ueki et al. (305). These workers analyzed the X-ray diffraction pattern of intact outer membranes of *E. coli* and *S. typhimurium*. The "equatorial" diffraction pattern, reflecting the distribution of electron density in the plane of the membrane, showed no signs of crystalline distribution of X-ray scatterers, which were shown to be porin trimers by the use of mutants. These are strong data, and in fact Rosenbusch himself has stated that the crystalline array is likely to be an artefact (252). However, Rosenbusch also advocates the presence of very small arrays which are thought to have a functional significance (260). This point is discussed in the next section.

DeMartini and Inouye (55) proposed that porin and lipoprotein formed a stoichiometric complex, because the dissociation of porin from peptidoglycan sheets was enhanced by the enzymatic cleavage, or mutational absence, of lipoprotein. However, currently available data do not support this proposal. First, if such a complex exists, the X-ray scattering pattern of the *lpo* mutants lacking the lipoprotein should be quite different from the wild type, because the main scatterer is the complex containing the porin trimer. Such a difference was never found (T. Ueki and H. Nikaido, unpublished data). Second, the association between porin and the peptidoglycan layer is likely to be an artefact (151), and the effect of lipoprotein in dissociating this artefact will have little physiological relevance. Third, the vesicles blebbed off from the outer membrane apparently contain large amounts of porin but little lipoprotein (111), suggesting that such an interaction, if it exists, must be very weak.

Permeability of Porin Channels: Molecular Aspects

Measurement of permeability. Several methods have been used to measure the permeability of the porin channel. These can be divided into methods that use in vitro reconstituted systems and those that use intact cells. Among the methods that use reconstituted systems, the method used in the early days was the liposome efflux assay of Nakae (184), in which the efflux of radiolabeled substances from liposomes containing porin was monitored by filtration of the liposomes through gel filtration columns. Although this method was instrumental in establishing the channel-forming properties of porin as well as the molecular-sieving properties of the channel, it has a very poor time resolution and is not of much use in the quantitative study of outer membrane permeability. In the second method one incorporates hydrolytic enzymes into the intraliposomal space and measures the rate of hydrolysis of external substrates by liposomes (302). This is a theoretically sound method, but in practice the difficulty of finding hydrolytic enzymes with the needed high activity, as well as the small volume/surface ratio of sonicated unilamellar liposomes, somewhat limits its utility. Some of the results obtained by this method may indeed by procedural artefacts (204). However, the latter difficulty appears to have been largely overcome by the addition of LPS to the reconstitution mixture (187), as LPS pushes the layers of liposomes apart due to its high negative charges, a phenomenon that leads to the production of unilamellar liposomes (185). In the third method, "liposome swelling assay," liposomes containing porin molecules within the bilayer are reconstituted so that large, impermeable molecules are contained in the intraliposomal space, and the rate of influx of solute molecules through the porin channel is determined from the initial rate of swelling of liposomes after their dilution into isotonic solutions of test solutes (144, 204, 208, 209). This method is simple, quantitative, and rapid and has been extremely useful. However, use of the method becomes quite difficult with charged solutes, because creation of membrane potential by differential diffusion of ions induces complex movement of buffer and other ions and because the method is totally nonspecific and responds to the flux of any ions and molecules. With extreme care and some compromise, diffusion of large anions can be measured with *E. coli* porin (204, 209), but so far measurement of the influx rates of large cations has not been possible. Although measurement of the diffusion rates of aminoglycosides, large cationic molecules, has been reported (183), we believe that the results are valid only in a qualitative way, if at all. Electrical methods of measuring the conductivity of planar lipid membrane or black lipid film containing porin have also been used extensively (22–25, 259, 260). The advantages and disadvantages of this method are the mirror image of those of the liposome-swelling method, in that the method is ideally suited for studying the diffusion of ions but not that of uncharged molecules.

Many methods have been used to study the properties of the channel in intact cells. In our opinion, only one method is rigorously correct in its principle. This is the method of Zimmermann and Rosselet (344), in which one measures the rate of hydrolysis, by intact cells, of compounds in the

medium. The compounds go through the porin channel before becoming hydrolyzed by enzymes located in the periplasmic space, and by combining Fick's first law of diffusion, which governs the first step, with the Michaelis-Menten equation, which determines the second step, one can calculate the permeability coefficient of the outer membrane. That the solutes are diffusing through the porin channel can be established by using mutants deficient in porin production (13, 211). Other methods usually involved determining the overall rates of transport of substrates or of hydrolysis of substrates and inferring the contribution of the porin channel from comparison with porin mutants or by the use of very low substrate concentrations. In other cases, the sensitivity of intact cells to antibiotics and other inhibitory agents has been used as a crude index of outer membrane permeability. These methods often give qualitatively sound results, but the results are usually impossible to interpret in a quantitative manner, because they are dependent not only on the permeability of the outer membrane but also on the V_{max} and K_m of the periplasmic enzyme or the cytoplasmic membrane-associated transport system. In some situations the results are largely limited by outer membrane permeability, but assuming that they are entirely limited by it and treating the results in a quantitative manner created many of the erroneous conclusions in this field, as discussed below. Another method that has been used is kinetic analysis of the influx or efflux of radiolabeled solutes from intact cells (33, 104). Such a method was tried earlier without result (G. Decad and H. Nikaido, unpublished data); we now understand why this was so. The half-equilibration time across the outer membrane, calculated directly from the permeability coefficients (see reference 210), is extremely short, usually much less than 1 s for small sugars, amino acids, or small peptides. What one observes with the time scale of 5 to 30 min has nothing to do with the outer membrane; it probably represents leakage into or out of the damaged cytoplasmic membrane of some cells.

Are the porin channels specific? Some studies with intact cells led to the conclusion that some of the porins are specific. For example, it was observed that the mutational loss of the OmpF porin did not reduce the rate of transport of various amino acids but decreased the rate of hydrolysis of nucleotides, and this result led some workers to argue that OmpF porin was a specific channel for nucleotides. As described by Nikaido et al. (206), such a conclusion is not warranted because the overall rates of these processes are influenced by the magnitude of the permeability barrier in relation to the size of the "sink" process, i.e., the V_{max} and K_m of the transport system located in the cytoplasmic membrane, or of the periplasmic hydrolytic enzyme (see Fig. 4). When the V_{max} of the latter step is low, even a greatly reduced permeability of the outer membrane would not affect the overall rate visibly, thus creating an erroneous impression that the particular porin lost by the mutation was not contributing to the transport process (Fig. 4).

More recently, the PhoE porin has been claimed to be specific for phosphate and phosphate-containing compounds (130). This is based on the observation that the overall rate of arsenate uptake was unchanged whether the sole porin present was PhoE or OmpF, but the rate of phosphate uptake was much higher in PhoE-containing cells than in OmpF-containing cells. However, the absolute rates were much lower with arsenate, and it is uncertain whether the outer membrane was really the limiting step in the transport of arsenate. In fact, liposome studies showed that the PhoE channel preferred any anionic compounds containing, for example, carboxylate or sulfate groups (see below), and the claim for phosphate specificity remains unconvincing.

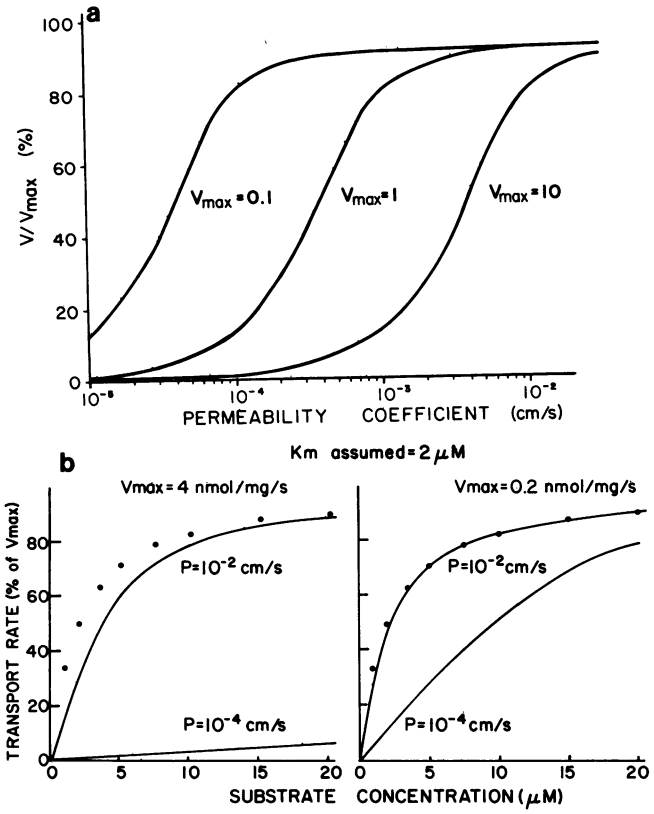

FIG. 4. Interplay of the outer and inner membranes in determining the parameters of active transport in intact cells. In (a) the solute is assumed to be transported by a system with a K_m of 1 μM and various values of V_{max} (nanomoles per second per milligrams of cells), located in the inner membrane. Assuming that the solute permeates through the outer membrane with permeability coefficient values shown, we show calculated velocities of the overall transport process relative to the V_{max} when the external concentration of the solute is 10 μM. In (b) dependence of actual transport rate on the external solute concentration is shown for cells containing an inner membrane active transport system with a K_m of 2 μM and the V_{max} values shown. The dots show the behavior of the system in the absence of the outer membrane barrier. With the "sink" of low V_{max} (right), lowering of the outer membrane permeability by a factor of 100 produces only minor effects on the overall rates of transport. Calculation in both parts was done by combining Fick's first law of diffusion with Michaelis-Menten kinetics (202, 344).

An intact-cell study concluded that the OmpC channel was specific for some peptides (104), but experiments in the laboratory of one of us could not confirm this claim (209). In conclusion, although we should not rule out the possibility of finding a true stereospecificity in porin channels, there is so far no convincing evidence, at least with the general porins OmpF, OmpC, and PhoE of *E. coli*.

Size of the pore. The liposome-swelling experiments showed that the rate of diffusion of uncharged solutes through the porin channels showed a strong dependence on the size, and hence the molecular weight, of the solute (208, 209) (Fig. 5). This is a feature expected for diffusion through small channels as predicted by Renkin (240), and one can approximate the nominal diameter of the pores by fitting the data to the Renkin equation. This procedure showed that the OmpF and OmpC pores of *E. coli* K-12 have diameters of 1.2 and 1.1 nm, respectively (209), and that the *P. aeruginosa*

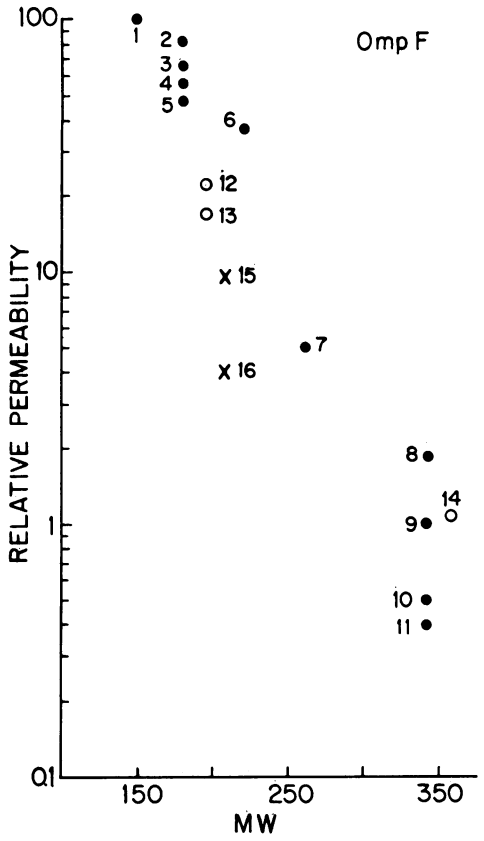

FIG. 5. Effect of solute size and charge on rate of diffusion of saccharides through the OmpF porin channel of *E. coli* K-12. The diffusion rates were determined by using the swelling of reconstituted proteoliposomes. The compounds studied were as follows: 1, L-arabinose; 2, D-galactose; 3, D-fructose; 4, D-mannose; 5, D-glucose; 6, *N*-acetyl-D-glucosamine; 7, 2,3-diacetamido-2,3-dideoxy-D-glucose; 8, lactose; 9, sucrose; 10, maltose; 11, melibiose; 12, D-gluconate; 13, D-glucuronate; 14, lactobionate; 15, D-saccharate (D-glucarate); and 16, D-mucate (D-galactarate). The symbols denote the type of solute: nonelectrolytes (●), monoanionic compounds (○), and dianionic compounds (×). The molecular weights (MW) of electrolytes shown are those of anions. Taken from reference 209.

PAO1 porin has an approximate diameter of 2 nm (339). However, these estimates cannot be very precise because there are a number of assumptions that cannot be totally correct. For example, the channel is assumed to be a straight hollow cylinder, but it is not (see above). The use of Poiseuille's law, applicable to macroscopic systems, for diffusion of individual molecules is certainly wrong. But at least the latter procedure does not produce too large an error because it is related to the viscous drag effect of the wall, which affects the final result only very slightly. In spite of the intrinsic weakness of this approach just mentioned, we believe that it is one of the more reliable methods, as we use the relative rates of diffusion of different solutes rather than the absolute rates and many unknown factors affecting the absolute rates may become cancelled out. We note that the value for the OmpF channel is in good agreement with the diameter of the negative stain-filled indentations measured in electron microscopy (282).

Another approach used was the single-channel conductivity obtained in the black lipid film experiments. The procedure appears to be very straightforward. One divides the single-channel conductivity by the bulk conductivity of the solution and then multiplies the value by the assumed thickness of the membrane; this gives the cross section of the pore. A value of 0.68 nm^2, or 0.9-nm diameter, was obtained for the *E. coli* OmpF channel (23). Although this value is in fairly good agreement with the estimate from the liposome experiments, it is difficult to assess the reliability of this estimate. The hydrated ions are passing through the channel as particles of a size comparable to that of the channel, and the use of bulk conductivity, in which there is no such steric hindrance to the movement of ions, is very difficult to justify. In fact, the estimated pore cross section shows a threefold difference depending on whether one uses salts of cations with tightly bound, large hydration shells such as Li^+ or those of cations with only a weak hydration such as Rb^+. Since the channel prefers cations over anions (24), it becomes even more difficult to justify the use of bulk conductivity because cations and anions make different contributions to conductivity in bulk solutions in comparison with the situation in the ion-selective channel.

In earlier studies in which efflux or influx of labeled molecules was determined after a set amount of time, it appeared as though there was a sharp molecular weight cutoff in the permeability of porin channels. Thus we found that solutes of <500 daltons nearly completely diffused through the outer membrane, with practically no flux of molecules of >700 daltons (54, 185). However, the liposome-swelling studies showed that there was a 100-fold (and even larger) difference in diffusion rates among the compounds well within the "exclusion limit" (209) (Fig. 5). Similarly, we believe that even the solutes above the exclusion limit will penetrate slowly through the porin channel, if given enough time and as long as the solute has a flexible structure. In other words, with flexible molecules there should be no sharp "cut-off"; the apparent sharpness of the cutoff previously observed was, we believe, an artefact of the particular procedure used.

Very recently S. A. Benson and A. DeCloux (personal communication) isolated mutants with altered OmpF and OmpC porins with apparently larger pore sizes. These mutants are able to transport maltodextrins of >1,000 daltons across the outer membrane, despite the absence of the LamB protein (see below), and are more sensitive than the wild type to a variety of antibiotics.

Effect of charge. Benz and co-workers (24) showed that the channels of "normal" *E. coli* porins OmpF and OmpC preferred cations over anions. Nikaido and associates (209, 210) showed, by the use of intact cells as well as reconstituted liposomes, that solutes carrying negative charges diffused through these channels more slowly than their uncharged counterparts and that solutes with double-negative charges diffused even more slowly. Acidic derivatives of glucose, gluconic and glucuronic acids, penetrated several times more slowly than glucose did, and a dicarboxylic acid derivative of glucose, glucaric acid, had an even slower penetration rate (Fig. 5). A similar preference for cations was also observed with *P. aeruginosa* porin (22).

Interestingly, the slow rates of influx of negatively charged solutes appeared to be more accentuated in intact cells of *E. coli*. Thus, although cephacetrile with one negative charge diffused at about one-half the rate of zwitterionic cephaloridine in liposomes, the difference was more than sixfold in intact cells. Perhaps in intact cells the influx of negatively charged solutes is further slowed down because of the presence of a Donnan potential that is negative inside (283), generated by the presence of polyanionic molecules, "membrane-derived oligosaccharides" of Kennedy (124).

Chemical modification of the NH_2 group of *E. coli* B porin trimer reduced its permeability to negatively charged solutes even further, and modification of the COOH group decreased the diffusion rates of positively charged solutes, as revealed by the use of liposomes (301). The loss of cation selectivity upon amidation of the carboxyl group has been confirmed in the black lipid film studies, which nevertheless could not confirm the effect of acetylation or succinylation of the NH_2 group (25).

In contrast to the situation described so far, the presence of negative charge does not hinder, and sometimes accelerates, the diffusion of solutes through the channel of PhoE protein (209), whose production is induced by phosphate starvation. The structure contributing the negative charge may be phosphate, sulfate, or carboxylate (209). The porin of *N. gonorrhoeae* also has a clear preference for anions (340); in this sense this porin resembles PhoE as well as the porin of mitochondrial outer membrane (47).

Effect of hydrophobicity. Zimmermann and Rosselet (344) first showed that the rate of penetration of β-lactam compounds through the *E. coli* outer membrane was affected by the apparent hydrophobicity of the solute molecule. Nikaido et al. (210) confirmed this conclusion and performed a more precise experiment. In contrast to the earlier work in which the apparent hydrophobicity was determined by the apparent partition coefficient of charged, sometimes zwitterionic and sometimes anionic compounds, they used only monoanionic molecules and used the partition coefficient of the uncharged form of the molecule to dissociate the effect of charge from the effect of hydrophobicity. The result was a uniform, monotonous, inverse dependence of diffusion rate on hydrophobicity: for each 10-fold increase in the octanol/water partition coefficient of the uncharged form of β-lactam, there was a roughly 5-fold decrease in the permeability coefficient. However, it seems unlikely that the same quantitative relationship would be found for smaller or larger molecules. In fact with the *P. aeruginosa* channel, in which the relative size of the β-lactam in comparison with the pore diameter is much smaller than in the *E. coli* channel, study with a limited number of β-lactams showed that the diffusion rate was much less dependent on the hydrophobicity of the solute (Nikaido, unpublished data). Although the molecular mechanism for the relative exclusion of hydrophobic solutes is not known, it may be because the entrance of hydrophobic molecules into the channel requires breaking relatively strong hydrogen bonds between the water molecules inside the channel and the groups lining the walls of the channel.

Our study with intact cells and β-lactams appeared to show that the wider OmpF channel and the narrower OmpC channel were affected similarly by the hydrophobicity of the diffusing solutes (210). However, experiments with reconstituted liposomes suggested that the penetration of hydrophobic peptides, for example, was more severely restricted by the narrower OmpC channel than by the OmpF channel. The ratio of diffusion rates in the OmpF versus OmpC channel was 3 with hydrophilic lysyl-aspartic acid (M_r = 261), but went up to more than 10 with the more hydrophobic threonyl-leucine (M_r = 232) (209). We believe that the results with intact cells containing the OmpC channel only (210) were inaccurate, because the values obtained in this experiment were close to the limit of significance.

The permeability of *S. typhimurium* porins toward β-lactams was studied in liposomes containing a β-lactamase (125). The permeability of the OmpF channel was the highest and that of OmpC was the lowest, with the OmpD channel showing intermediate values.

Closing and opening of the channel. Schindler and Rosenbusch (259, 260) showed that the porins, inserted into planar bilayers of phospholipids, apparently could close the channel if an electrical potential exceeding a certain threshold value was applied across the bilayer and that the porin channel therefore was a voltage-controlled channel. Such a phenomenon is known also for the mitochondrial porin, which is often referred to as VDAC, or voltage-dependent anion channel (47). It is difficult to assess the physiological significance of this phenomenon. In the early experiments of Schindler and Rosenbusch (259), a voltage of 140 mV was required to close the pores, and one could argue that such a high potential was unlikely to exist across the outer membrane, although Donnan potentials of up to 80 mV could be created if the cells were in a medium of low ionic strength (283). In a more recent study, Schindler and Rosenbusch (259) have shown that the threshold voltage could become as low as 40 mV, depending on the nature of the porin preparations used; this observation suggests that the physiologically present Donnan potentials may close the pore. However, the planar membrane reconstituted with the native outer membrane, presumably the system best reflecting the native state of the porin, still requires about 130 mV for channel closure (259), and another laboratory (using the black lipid film technique) has consistently failed to observe the voltage-dependent closure of porin channels (23, 24). We have tried to see if the Donnan potentials could close the channel in intact cells by measuring the rate of hydrolysis of cephaloridine by a periplasmic β-lactamase (J. Hellman and H. Nikaido, submitted for publication). The results suggest that Donnan potentials as high as 80 mV produce practically no effect on the permeability of the outer membrane.

In *P. aeruginosa*, evidence suggests very strongly that most of the porin channels are closed. The organism is intrinsically resistant to a wide variety of antibiotics, and its outer membrane was indeed shown to have permeability at least 100-fold lower than that of *E. coli* in studies with intact cells (6, 338). Reconstitution of purified porin into liposomes showed that the neutral sugars diffused into liposomes at rates far lower than in liposomes containing comparable amounts of *E. coli* porin (339). Although this can theoretically be due to the low permeability of an individual channel, reconstitution into black lipid films showed that the single-channel permeability was quite high, as expected from the larger exclusion limit (22). Clearly the simplest hypothesis to explain all of these observations is that most of the porin channels are closed. It is not clear whether this represents a reversible phenomenon. Application of membrane potential (22), or a limited attempt at nutrient starvation (338), has failed to produce significant opening of these channels.

As described earlier, porins interact strongly with LPS, and suggestions have been made that this interaction has a profound influence on the function of the porin. Schindler and Rosenbusch (259) have observed that porins devoid of LPS can produce only very unstable channels and that LPS was necessary to keep the channel open and to produce small clusters of porin. However, such an effect was not observed with the liposome system (E. Y. Rosenberg and H. Nikaido, unpublished data). In both *E. coli* and *P. aeruginosa*, antibiotic-hypersensitive mutant strains have been reported to produce LPS of altered structure (see below), and such LPS has been assumed to keep the channel in an open state. Although this is a very attractive hypothesis, as yet we have no solid evidence showing that the association of altered LPS indeed changes the fraction of open porin channels.

TABLE 1. Predicted parameters for transport of solutes in *E. coli*[a]

Permeability coefficient (P) (μm/s)	Example[b]	External concn (μM) giving half-maximal rate	
		High V_{max} system[c]	Low V_{max} system[d]
300	Glycerol	1.5	1.0
25	Glucose	7	1.3
1	Lactose	150	8.5

[a] Under certain conditions, the diffusion of solutes through the outer membrane becomes partially limiting in the overall transport process, and thus the transport parameters measured in intact cells will not correspond exactly to the kinetic parameters of the transport system in the inner membrane. The table shows external concentrations of solutes that will give half-maximal rates of transport in intact cells, calculated by assuming that the solute is transported across the inner membrane by a system with a K_m of 1 μM.

[b] Example of solutes that would have the permeability coefficients specified in column 1 (see reference 208).

[c] V_{max} assumed = 4 nmol mg^{-1} s^{-1} as in systems typically transporting carbon sources. For example, V_{max} values for transport of glucose and lactose are 6 and 5 nmol mg^{-1} s^{-1}, respectively (126). V_{max} for uridine is also quite high (2 nmol mg^{-1} s^{-1}) (126).

[d] V_{max} assumed = 0.2 nmol mg^{-1} s^{-1} as in systems typically transporting growth factors. For example, V_{max} values for valine, leucine, and proline are 0.4, 0.2, and 0.3 nmol mg^{-1} s^{-1}, respectively (126).

In view of the strong and functionally important interaction between the LamB pore-forming protein and the soluble maltose-binding protein in the periplasm (see below), it is tempting to assume that a similar interaction exists between the porins and the periplasmic binding proteins. In fact Lo and co-workers (139, 140) reported that porins interacted with the dicarboxylate-binding protein on the external surface of the outer membrane to accelerate the diffusion of succinate through the porin channel. This is a fascinating idea, but the results are not yet totally convincing. So much depends on the use of antibody against the succinate-binding protein, which was purified by single-step affinity chromatography. Yet the procedure for the synthesis of the affinity matrix suggests that the carrier was linked to a random copolymer of aspartate rather than to single aspartate residues, which these workers expected to act as an analog of succinate (see reference 139).

Permeability of Porin Channels: Physiological and Ecological Aspects

Diffusion through the outer membrane is a simple, passive diffusion process. The rate of this process is determined by Fick's first law of diffusion, $V = P \times A \times \Delta c$, where V is the rate, P is the permeability coefficient of the membrane toward the given solute, A is the area of the membrane, and Δc is the difference in the concentrations of the solute across the membrane. P will be different for different solutes; we have seen that there is a nearly 100-fold difference between arabinose and lactose. It is also influenced by the nature of the porins present, OmpF, OmpC, or PhoE in *E. coli*, for example. Even when all of these factors are kept constant, we see that V changes linearly with Δc; this is one of the most important facts to keep in mind in considering diffusion through the outer membrane. The periplasmic concentrations of various nutrients are expected to be quite low owing to the presence of powerful active transport systems located in the cytoplasmic membrane, and thus as a first approximation we can say that, for a given nutrient, V is more or less proportional to its concentration in the external medium. That is, when the concentration of carbon source, for example, glucose, drops form the very high level we use in the laboratory (for example, 0.5% = 28 mM) to very dilute concentrations thought to be ecologically relevant (126), such as 1 μM, there will be a more than 10,000-fold reduction in the diffusion rate of glucose across the outer membrane.

Now what is important for bacterial physiology is the magnitude of V in relation to the magnitude of the V_{max} of the cytoplasmic transport system. If the former becomes significantly lower than the latter, the active transport system will not be able to function to its full capacity. In Table 1 we show the external concentrations of nutrients at which the overall rate of transport will become one-half of the V_{max}, i.e., the concentrations at which the outer membrane diffusion would become a strongly limiting process for different compounds and for active transport systems with different throughputs or V_{max} values. As seen, if the V_{max} of the active transport system is small, the outer membrane is not a significant barrier until the external concentration falls to the micromolar range (see also Fig. 4). In contrast, the outer membrane permeability could become a serious problem for high V_{max} systems, such as those for the transport of carbon sources (see footnotes to Table 1), or when the solute becomes larger, more hydrophobic, or negatively charged. We believe that this is why the active transport system for lactose has an unusually high K_m, reported to be 70 to 900 μM, in comparison to most other transport systems of *E. coli* characterized by low micromolar K_m values: a higher affinity would be wasted since the outer membrane diffusion would become nearly completely limiting in micromolar concentrations of solutes in the external medium. This is also why *E. coli* needs a specialized outer membrane transport channel, the LamB protein (see below), to carry out the transport of maltose with a very high affinity, with an overall K_m of around 1 μM.

These considerations also explain the danger of conclusions from simple qualitative observations. The observation that "porin-deficient" mutants grew with a normal generation time in ordinary laboratory media did not negate the importance of the porin pathway in nutrient transport, because carbon sources are present usually in millimolar concentrations in such media (see above), and Fick's first law of diffusion tells us that the bacterial cell will be able to tolerate 1,000-fold or greater reductions in P in the presence of such a high value of Δc. The observation that loss of certain porin species did not affect the transport of certain solutes did not indicate the presence of specificity: some of these solutes happened to be transported by systems with low V_{max} values, and under these conditions the overall transport process is unlikely to be affected by the outer membrane.

It is also necessary, in understanding the role of outer membrane barrier in antibiotic resistance, to consider the balance between the influx rate of the agent across the outer membrane and the rate of its removal from the periplasmic space. This is particularly important with β-lactams, which are removed by β-lactamases that appear to be present in the periplasm of practically all gram-negative bacteria (291). The role of the outer membrane barrier in β-lactam resistance has been discussed in detail elsewhere (Nikaido, Pharmacol. Ther., in press).

The presence of multiple species of porins in enteric bacteria gives us a unique opportunity to examine the roles porins play in the physiology of the organism. As stated earlier, OmpC pore is slightly narrower than the OmpF pore. This produces only a small (about twofold) difference in the

permeability of small solutes such as arabinose or glucose, but a much larger difference in the penetration rates of larger, hydrophobic, or multiply negatively charged compounds (209). One would predict that bacteria containing only the OmpC porin would acquire a low-level resistance to various noxious agents, which tend to have the characteristics just mentioned. Thus eliminating the OmpF porin would indeed be an ingenious mechanism for creating low-level resistance, as the bacteria can cut down the diffusion rates of these noxious molecules drastically without much reducing the permeability to most of the nutrients. In fact, the *E. coli* mutants lacking the OmpF porin are somewhat resistant to tetracycline (236), chloramphenicol (238), and β-lactams with intrinsically low penetration rates (98).

In *E. coli* as well as *S. typhimurium*, synthesis of the OmpF and OmpC porins is controlled by osmotic pressure (99, 279) as well as by temperature (150). Thus inclusion of 10% sucrose in the growth medium, in addition to raising the growth temperature to 42°C, represses the production of OmpF porin. The molecular mechanisms of these regulatory processes have been studied extensively (153, 173). We can imagine that the major purpose of these processes is to let the bacteria "sense" that they are in the bodies of animals, in which the osmotic pressure of the environment is quite high (0.9% NaCl has an osmotic pressure nearly equivalent to 10% sucrose) and the temperature is also high. In the presence of these "signals" the bacteria then produces only the narrower OmpC porin. This would be beneficial to the bacteria in protecting them from some of the inhibitory substances present in the bodies of animals. In fact, we have recently found that a strain of *S. typhimurium* originally producing both OmpF and OmpC porins mutated to become OmpC deficient in the body of a patient receiving a β-lactam, cephalexin (A. Medeiros and H. Nikaido, in preparation). The mutant, when grown in low-osmolarity medium, does produce OmpF porin and is not any more resistant to β-lactams than the parent. However, it is much more resistant than the parent in media containing 1% NaCl, which almost completely represses the production of OmpF porin. That this mutant was selected in the patient's body clearly indicates that the parent strain was living with only the OmpC porin in this environment.

The production of OmpF porin in a low-osmolarity and low-temperature environment suggests that it is the porin used by these enteric bacteria when they have to survive outside the bodies of warm-blooded animals, e.g., in ponds and rivers, as was originally proposed by M. J. Osborn (personal communication). The wider diameter of the OmpF pore would certainly be advantageous in accelerating the assimilation of nutrients from very dilute environments by increasing the permeability coefficient, P, as we have seen earlier.

SPECIFIC DIFFUSION CHANNELS

LamB Protein or a Channel for Maltose and Maltodextrins

In 1975, it was shown that *E. coli* mutants with defective LamB protein, an outer membrane protein hitherto recognized as the receptor for phage lambda, were impaired in the active transport of maltose when its external concentration was lower than 10 μM (101, 292). Several years later, reconstitution with purified LamB protein has shown that it can indeed produce transmembrane diffusion channels (26, 144, 186). Because of this property, and because the protein shares many characteristics with the nonspecific porins, it is sometimes called "maltoporin." All laboratories agree that the channel has some nonspecific element, as it allows the diffusion of amino acids and unrelated sugars (144, 186) and alkali metal cations (26). Furthermore, there is agreement also on the presence of some substrate specificity in the channel. Luckey and Nikaido (144) showed, by using the liposome-swelling assay, that the LamB channel is not very discriminating among monosaccharides, but becomes more and more discriminating for larger and larger saccharides, and that it is almost completely specific at the level of trisaccharides (for example, maltotriose diffusing at least 100 times faster than trisaccharides of unrelated structure such as raffinose or melezitose). Furthermore, they showed that the diffusion of glucose through the LamB channel could be inhibited by the addition of higher oligosaccharides of the maltose series, and a K_i of about 1 mM was found with maltoheptaose (145). This result shows the presence of a specific binding site in the channel. A similar conclusion was obtained also by Ferenci and co-workers (68), who showed, in an ingenious approach, the binding of maltodextrin and starch to the LamB protein on the surface of intact *E. coli* cells. Although Nakae (186) reported initially that the LamB channel was totally nonspecific on the basis of data obtained by the liposome efflux assay, his group (187) later used vesicles containing amyloglucosidase or yeast α-glucosidase in the intravesicular space and came to the conclusion that the LamB channel was far more permeable than the porin channel for maltotriose and larger oligosaccharides of the maltose series.

There is, however, one controversial point. Nakae and Ishii (187) found that the *E. coli* B porin and *E. coli* K-12 LamB channels show approximately the same degree of permeability to maltose by using liposomes containing amyloglucosidase. In contrast, Nikaido and co-workers reported, for the *E. coli* K-12 LamB channel, at least an order of magnitude higher permeability for maltose than for the *E. coli* K-12 OmpF porin, on the basis of the results of a swelling assay (compare the swelling rates reported in reference 144 with those in reference 208).

It was conceivable, a priori, that this difference reflected some fundamental differences in the assay methods used. However, after the repetition of experiments in the two laboratories (G. Nikaido and H. Nikaido, unpublished data; T. Nakai, personal communication), the simplest explanation seems to be that a misleading impression was created in the comparison of proteins from two different sources (187) because of the unusually high permeability of the *E. coli* B porin. If so, the LamB channel indeed seems to possess properties optimized for the transport of maltose and maltodextrins.

There are many pieces of evidence suggesting that LamB protein interacts physically with the maltose-binding protein (MBP) of the periplasmic space and that this interaction is important in the function of the channel. For example, Wandersman et al. (327) isolated several mutations in *malE*, the structural gene for MBP. Although MBP from the mutants had a somewhat lower affinity toward maltodextrins, the alteration was minor and could not explain the complete inability of the mutants to grow on maltodextrins. These results suggested that the mutants were defective in the transport of maltodextrins across the outer membrane. This suspicion was confirmed by an elegant competition experiment, and it was concluded that interaction of MBP with the LamB protein is necessary for the efficient transport of maltodextrins through the LamB channel. This presumed interaction was then demonstrated by Bavoil and Nikaido (12), who showed that LamB protein suspended in Triton

X-100 was specifically adsorbed to an MBP-Sepharose column. Furthermore, one of the *malE* mutant proteins, predicted by Wandersman et al. (327) to be deficient in the interaction with LamB protein, indeed failed to adsorb the wild-type LamB protein (15). Finally, Neuhaus et al. (195) have recently reported that MBP binds to LamB protein in a planar lipid bilayer with a K_d of 0.15 μM, with striking effects on the permeability of the channel.

How does the interaction between MBP and LamB protein facilitate the transport function of the LamB channel? We may recall here that the substrate for diffusion through the LamB pore can be quite large, up to 1,152 daltons (maltoheptaose). The channel does not appear to be exceptionally large (26, 144, 195), and thus it seems likely that the oligosaccharide molecule is aligned perpendicular to the plane of the membrane, perhaps through its specific binding to the site within the pore. Conceivably one may need an extra driving force for a rapid diffusion of such a molecule in addition to the concentration difference, because the binding will tend to slow down the process.

Although it appears certain that MBP is needed for the efficient transport of maltodextrins through the LamB channel, it is unclear at present whether a similar requirement exists also for the transport of maltose. The swelling assay (144) and an assay with amyloglycosidase-containing liposomes (Nikaido and Nikaido, submitted for publication), as well as experiments with black lipid films (26) and planar bilayers (195), suggest that LamB forms an open channel which appears to have high permeability to maltose according to the first two studies. On the other hand, some results on *lamB* missense mutants present a different picture. Many of these mutants show reduced rates of maltose diffusion across the outer membrane (292); yet when the mutant LamB protein was reconstituted into the proteoliposomes, the rate of influx of maltose and maltotriose was quite normal (146). However, two mutant LamB proteins least active in the intact cell transport assay were found to be defective in their interaction with the immobilized MBP (146), suggesting that the interaction with MBP is crucial in the transmembrane transport of maltose. Possibly MBP is not mandatory but enhances the diffusion of maltose.

The LamB protein behaves as a rather nonspecific pore in reconstituted systems, as seen above. It also can behave as a nonspecific pore in intact cells, as was first shown by von Meyenburg and Nikaido (326). Although glucose diffuses nearly three times faster than maltose in liposome systems (144), in intact cells it seems to diffuse through the LamB channel only very slowly, at a rate estimated to be less than 2 to 10% of that through the porin channel (12). These observations led to the concept of "plugging," in which MBP, associated with the periplasmic face of the LamB protein, inhibits the influx of any substrate that cannot bind to MBP. This idea received support from the observations of Neuhaus et al. (195) that MBP closes the LamB channel for the diffusion of Li^+ and Cl^- in planar phospholipid bilayers, when voltage in the "physiological" direction, i.e., negative on the side containing MBP, was applied. It is also supported by the observation of Heuzenroeder and Reeves (107) that the overall rates of active transport of lactose and mannitol were higher in a *malE* mutant than in the *malE*$^+$ strain in a background lacking porin but containing the LamB protein. The difference observed, however, was rather small. More recently, Brass et al. (submitted for publication) found that the presence or absence of MBP produced no difference at all in the rate of transport of lactose through the LamB channel.

The *lamB* gene has been completely sequenced (41), and the deduced amino acid sequence suggests the mature protein of 47,393 daltons with the NH_2-terminal extension of 25 residues as the leader sequence. The amino acid composition is not particularly hydrophobic. This protein resembles porins in many ways: it forms a stable trimer (227) rich in β-sheet structure (80), with a noncovalent association with the peptidoglycan layer (75). Analysis of missense mutants by DNA sequencing, as well as the secondary-structure prediction, suggests that the polypeptide may weave across the thickness of the lipid bilayer many times, most of the time using the β-sheet regions to cross the membrane (35). Since the protein has been crystallized, the results of X-ray crystallographic studies (80) are eagerly awaited. Recently, Ferenci and Lee (67) devised a method of isolating *lamB* mutants with altered specificities by using an affinity column selection of intact cells; this ingenious method will be useful in the study of structure-function relationships in this interesting protein.

Other Proteins Involved in Specific Transport Processes

T6 receptor or Tsx protein is known to be involved in the diffusion of nucleosides across the outer membrane (96). Although nucleosides have sizes that allow their diffusion through the porin channel, the rates should be quite slow due to its size, and the specific channel is required because of the exceptionally high V_{max} of the nucleoside active transport system of *E. coli* (126). Successful reconstitution of Tsx protein has not been reported, but it is likely to be a channel-forming protein in view of the fact that its function does not require the presence of TonB product, in contrast to the other transport systems described below. Indeed, Heuzenroeder and Reeves (108) reported that the Tsx protein allows the diffusion of serine, glycine, and phenylalanine, but not glucose. Since the uptake of glucose proceeds with a much higher V_{max} in comparison with the transport of amino acids, the failure to detect the stimulation of glucose uptake could simply be due to a technical difficulty.

E. coli outer membrane contains several more proteins that are involved in specific transport processes. At least some of these proteins bind their substrates quite tightly, in contrast to the very loose or nondemonstrable binding seen with the LamB and Tsx proteins. For the proper function of all of these proteins, functional TonB product is required (see reference 231). Although TonB is often assumed to be involved in "energy coupling," its mode of action is not clear. Gene *tonB* has been sequenced, and the amino acid sequence is very unusual, containing 16% proline residues and stretches such as Glu-Pro-Glu-Pro-Glu-Pro-Glu-Pro-Ile-Pro-Glu-Pro and Lys-Pro-Lys-Pro-Lys-Pro-Lys-Pro-Lys-Pro-Lys-Pro (231). It was reported that the collaboration between TonB and the outer membrane protein BtuB resulted in the accumulation of very large numbers of vitamin B_{12} molecules in the periplasmic space against the concentration gradient (244), but the mechanism proposed in a recent paper (113) from the same laboratory appears inadequate for explanation of these results. Protein BtuB has a molecular weight of 60,000, serves as the receptor for E-group colicins as well as phage BF23, and binds vitamin B_{12} very tightly, with a K_d of 3 nM (for references, see reference 113).

There are a number of outer membrane proteins involved in the transport of various chelates of ferric ion. (The transport of iron chelates in bacteria has been discussed thoroughly in a recent review by Neilands [193].) The TonA

(or FhuA) protein, necessary for the uptake of ferrichrome, has a molecular weight of 78,000 and serves as the receptor for phages T1, T5, φ80, and colicin M. Ferrichrome appears to have a high affinity for this receptor on the basis of its interference with the adsorption of phages (147, 328), but the TonA protein has not been purified. More recently, mutations in gene *fhuB* were found to abolish utilization of not only ferrichrome but also all other ferric hydroxamate siderophores; an intriguing observation is that inner membrane vesicles of *fhuB* strains were not defective in ferric hydroxamate transport (234). Although this suggests that FhuB might be involved in transport across the outer membrane, its subcellular location is currently unknown. The FepA protein, required for the transport of ferric enterochelin (enterobactin) across the outer membrane, is an 81,000-dalton protein, and crude extract of FepA protein was shown to bind the ferric enterochelin (112). The FepA protein has been purified and characterized, but the molecular mechanism of transmembrane transport remains unknown (193). *E. coli* produces, in addition, an 80,500-dalton protein believed to be ferric citrate receptor and a few other outer membrane proteins induced by iron starvation, but their properties are largely unknown (193).

Specific Transport Proteins from Nonenteric Bacteria

Mizuno and Kageyama (174) found that growth of *P. aeruginosa* in media containing glucose induced the production of outer membrane protein D. Hancock and Carey (93) showed that protein D actually contained two proteins, D1 and D2, and that the glucose-inducible protein was D1. They purified D1 and showed it to form ion-permeable channels upon reconstitution into the black lipid membrane. It is likely that D1 is a channel with some specificity toward glucose, somewhat similar to the maltose preference of the LamB channel discussed above, but the presumed substrate preference of this channel remains to be demonstrated.

P. aeruginosa produces a 48,000-dalton protein, P, upon phosphate starvation (95). This protein resembles the *E. coli* porins in producing a trimer which is resistant to the denaturing action of SDS (7). Upon reconstitution into black lipid films, it produced a channel with a very strong anion selectivity and a much lower single-channel conductance than the porin. The channel indeed allowed the diffusion of F^- and Cl^-, but showed very low conductivity toward phosphate (95). Nevertheless, it is reported that the permeability conferred would be sufficient for phosphate uptake, given the large amount of protein P incorporated into the outer membrane (95).

LIPID BILAYER AS A DIFFUSION BARRIER

The Asymmetric Bilayer

There is little doubt that lipid bilayer forms the basic continuum of the outer membrane just as in most other biological membranes. This is suggested by the typical trilaminar morphology of the thin section of outer membrane (see reference 84 for review), the X-ray diffraction data showing the 0.42-nm spacing between the hydrocarbon chains in directions parallel to the surface of the membrane (305), the cooperative melting of quasicrystalline packing of lipid hydrocarbon chains, a phenomenon characteristic of bilayer lipids (221), and the cleavage of the membrane in the middle by a freeze-fracturing procedure (69, 278, 321).

In enteric bacteria, most of the LPS molecules appear to be located in the outer leaflet of the bilayer, as shown by electron microscopy after antibody labeling (179, 181) and by enzymatic modification of LPS in intact cells (73). In view of the evidence for a strong interaction between LPS and phospholipids (for example, see reference 72), it was generally assumed that LPS and phospholipids formed mixed bilayers. Smit et al. (278), however, noted that the number of hydrocarbon chains of LPS present in the outer membrane of a single cell is approximately the same as the number of hydrocarbon chains in phospholipids of the outer membrane. The average area values for hydrocarbon chains used by these authors may have to be revised, but in the first approximation one can assume that a hydrocarbon chain of both LPS and phospholipid will occupy an area of a similar size. If so, these analytical results suggest strongly that the outer leaflet of the bilayer is almost entirely free of phospholipids, as the use of many phospholipids for the construction of the outer leaflet will produce a shortage of the lipids that have to cover the inner surface of the membrane. This idea was tested by Kamio and Nikaido (121) by treating intact cells of *S. typhimurium* with a macromolecular labeling reagent, cyanogen bromide-activated dextran. If there had been any phosphatidylethanolamine molecules in the outer leaflet of the outer membrane, the exposed ethanolamine head groups should have been labeled with dextran, but this did not occur (Fig. 6A). (A similar result was obtained by Schindler and Teuber [263] with dansyl chloride as the labeling reagent. Such a reagent may be expected to penetrate through the lipid bilayer of the usual biological membranes, but with the outer membrane the lipid bilayer has an unusually low permeability to hydrophobic molecules [200] and porin channels also have low permeability to bulky hydrophobic molecules [see above].) As a positive control, the outer membrane of "deep rough" LPS mutants (Fig. 2) was shown to contain much larger amounts of phospholipids, some of which were apparently located in the outer leaflet and reacted with the labeling reagent (Fig. 6B). Since the deep rough LPS contains very short saccharide chains, the effect might be thought to be due to the "unshielding" of neighboring phospholipid head groups. However, such an interpretation is unlikely for several reasons. (i) The labeling of phospholipid head groups did not occur with mutants with an Rc-type LPS (see Fig. 2), whereas it occurred almost to the full extent with Rd_1 mutants (Fig. 2). Since the LPS saccharide lengths differ by only one sugar residue, it is very difficult to imagine that a single sugar residue in Rc LPS could have provided a full shielding effect. (ii) The labeling was observed with mutants with apparently normal LPS but with reduced amounts of outer membrane proteins and increased amounts of outer membrane phospholipids (121).

Other workers have reported that stationary-phase cells of deep rough mutants contained up to five or six times more LPS per unit area of the membrane in comparison with the wild type and concluded that a radically different organization was necessary to accommodate these huge amounts of LPS (85–87). Repetition of their work with exponential-phase cells of the same mutant strains of *S. typhimurium* showed (J. Goldberg and H. Nikaido, unpublished data) that the content of LPS increases in deep rough mutants by only 20 to 30%, confirming the results of Havekes et al. (100) but showing nothing similar to the results of Gmeiner's group (85–87). Furthermore, it is difficult to imagine what happens to the large amount of overproduced LPS. Gmeiner's suggestion (85–87) that proteins and LPS molecules become further compressed seems unreasonable, as proteins cannot be compressed so drastically and the average area per hydrocarbon in membrane bilayers is not so far from the

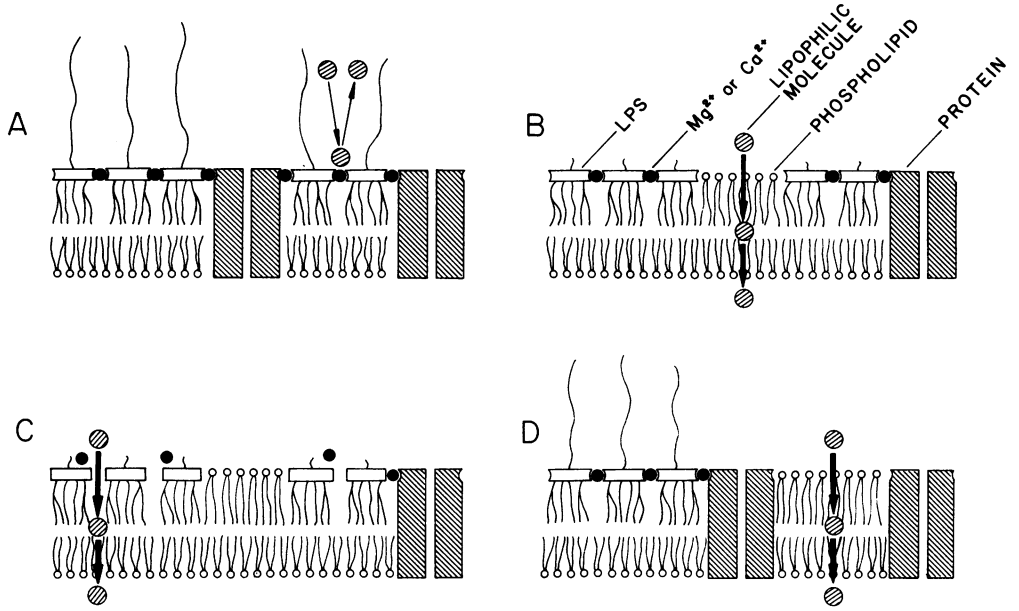

FIG. 6. Hypothetical structure of outer membrane in the wild type, deep rough mutants, and EDTA-treated wild-type cells of *E. coli* and *S. typhimurium*. (A) Wild-type strain in which the outer leaflet is almost entirely composed of LPS and proteins. (B) Deep rough mutants. Hydrophobic molecules are assumed to penetrate through the phospholipid bilayer domains. (C) Deep rough mutants. Hydrophobic molecules are assumed to penetrate through the LPS domains, which could have become more permeable due to alterations in LPS structure. (D) EDTA-treated wild-type cells. Phospholipid molecules are assumed to fill the void left by the selective removal of LPS by EDTA.

area in a maximally compressed structure. Perhaps the most likely explanation is that in the stationary-phase culture used by Gmeiner's group there were large numbers of outer membrane blebs or protrusions; the blebbing is enhanced in deep rough mutants, especially in the stationary phase (J. Smit and H. Nikaido, unpublished data).

If the LPS and phospholipids are completely segregated into the outer and inner leaflets in the outer membrane, one would expect the phospholipids to show the fluidity and melting behavior of a pure phospholipid monolayer, without much influence from the LPS. This is precisely what was found by Nikaido et al. (212) by using spin-labeled fatty acids as probes. In contrast, various other studies using the spin label technique (255), X-ray diffraction (65), and ^{2}H-nuclear magnetic resonance (78, 198) all showed that either the outer membrane lipids melt at higher temperature than the phospholipids of the cytoplasmic membrane or they are more restricted in motion than the cytoplasmic membrane lipids. However, in these studies outer membrane free of peptidoglycan layer has been used, and it is known that redistribution of LPS (and probably phospholipids) occurs in such a preparation, producing leaflets containing both LPS and phospholipids (179). Thus the restricted motion of the outer membrane phospholipids is very likely to be an artefact, and results of differential scanning calorimetry strongly support this idea (170). In this study, whole cells as well as the isolated cytoplasmic membranes of wild-type *E. coli* showed only one thermal transition attributable to lipid melting in the range of −5 to 10°C, whereas cells previously heated at 100°C showed an additional transition centered around 25°C, which was also the transition seen in the isolated outer membrane. Because the intact cells did not show the 25°C transition, it is reasonable to assume that the outer membrane phospholipids in intact cells were melting at the same temperature range as the inner membrane lipids, i.e., between −5 and 10°C. When the cells are boiled, or when the outer membrane free of peptidoglycan layer is prepared, the segregation of LPS and phospholipids into the two leaflets may be largely lost, and the lipids in the mixed phospholipid-LPS bilayers may undergo thermal transition at a higher temperature because of the immobilizing effect of the neighboring LPS molecules. In fact, in the study of Melchior and Steim (170), the magnitude of −5 to 10°C transition seems to decrease after the 100°C treatment of whole cells, a result expected if outer membrane phospholipids contributed to this transition in unheated cells. Thus, in conclusion, the outer membrane phospholipids seem to show fluidity and melting behavior similar to cytoplasmic membrane lipids if the experiments are done correctly. It is regrettable that so much effort has been wasted by using peptidoglycan-free outer membrane, in spite of the fact that such membranes have a radically altered organization, as was clearly shown by Mühlradt and Golecki (179).

The outer leaflet containing LPS seems to show much higher viscosity in the hydrocarbon region (212). The data on the cooperative melting behavior of LPS, however, are rather difficult to interpret. By using spin-labeled fatty acid probes, Nikaido et al. (212) could not detect any well-defined transition point with LPS bilayers. In contrast, X-ray diffraction (65) and light-scattering (317) studies with outer membrane or isolated LPS or both have detected quite a sharp phase transition centered around 25°C. One possible explanation of this discrepancy is that Nikaido et al. (212) measured thermal behavior in the presence of 10 mM Mg^{2+}, whereas other studies were done in the absence of divalent cations; indeed the X-ray diffraction study (65) showed that even 0.1 mM Mg^{2+} abolished the thermal transition completely. Such a strong effect of divalent cations is expected from the fact that divalent cations alter the melting behavior of acidic phospholipids in a dramatic manner (228). In the intact outer membrane, large amounts of divalent cations are present presumably in association with LPS. If so, this transition at 25°C should not occur in intact cells, and this was precisely what was observed by Melchior and Steim

(170) by the use of calorimetry. EDTA is usually used in the isolation of outer membrane free of peptidoglycan layer, and perhaps the appearance of a 25°C transition in the isolated outer membrane may be related to the removal of divalent cations by EDTA; from the available data it is impossible to decide whether the 25°C transition seen by Melchior and Steim (170) is due to LPS or phospholipids immobilized by LPS (see above) or both.

The outer membrane can be fractured in the middle, a result supporting the bilayer structure of the lipid continuum. The concave face of the membrane is filled with particles. Because the density of the particles showed a good correlation with the protein content of the outer membrane, it was hypothesized that these particles were largely proteinaceous in character (141, 278). More recently, it has been argued that the particles are mostly composed of LPS and that the role of proteins is simply to "induce" the aggregation of LPS (151, 322), mostly on the basis of the observation that pure LPS bilayers and outer membrane of mutants lacking most major proteins could be induced to produce particles of "similar shape" under certain nonphysiological conditions. We do not believe that it is fruitful to discuss this controversy further, but we note that, with particles of this small size (8 to 10 nm), shadowing after freeze-fracture does not reveal any details of structure, and it is difficult to argue, from morphology alone, that particles seen under one condition are of the same nature as those seen under different conditions.

The segregation of LPS and phospholipids seems nearly complete in enteric bacteria such as *E. coli* and *S. typhimurium*. As described below, this totally asymmetric structure appears to be essential for these bacteria that live in an environment containing high concentrations of the detergent cholates. For some nonenteric bacteria, in contrast, there may exist little need to make the outer membrane impermeable to hydrophobic substances, and it seems possible that they produce more symmetrical bilayers with significant numbers of phospholipids also in the outer leaflet. Nevertheless, the available data appear to be more easily interpreted by assuming the asymmetric bilayer (see below). Possibly the absence of phospholipids in the cell surface is important in protecting the bacteria from ubiquitous phospholipases.

Interaction Between LPS Molecules

What produces the segregation of LPS and phospholipids into separate leaflets? The process appears especially difficult because LPS is synthesized in the cytoplasmic membrane, where it is inserted in the phospholipid bilayer (254), and because LPS and phosphatidylethanolamine have been reported to interact very strongly, even in a stoichiometric manner (72). However, Takeuchi and Nikaido (295) have shown, by using spin-labeled phospholipids reconstituted with LPS, that LPS-LPS interaction (and possibly phospholipid-phospholipid interaction) is very strong at least in the presence of Mg^{2+} (see below for the possible significance of this condition) in comparison with LPS-phospholipid interactions and that domains containing pure LPS are almost completely stable in bilayers containing LPS and phospholipids in "physiological ratios." This inherent stability of LPS-only domains will obviously contribute to the stabilization of the segregation of LPS and phospholipids. Although this result appears to contradict the results of Mühlradt and Golecki (179) showing the rapid scrambling of the outer membrane components after the dissociation of outer membrane from peptidoglycan, the contradiction is only apparent because the stable "domains" of LPS and phospholipids detected by the electron-spin resonance technique may be quite small. It may be important that Mg^{2+}, which was present in the study of Takeuchi and Nikaido (295), was either not added or was removed by EDTA in the work of Mühlradt and Golecki (179). Also, there may be no real discrepancy between these results and those of Rothfield's group (72), because the latter relied heavily on monolayer methods, in which the lateral interactions between the lipid molecules may play only a minor part in determining the organization of molecules.

The proximal portion of the saccharide chain of LPS has a number of negatively charged groups (Fig. 2), and the juxtaposition of LPS molecules is expected to result in a strong electrostatic repulsion between the molecules. Indeed, the counter ions or cations play a crucial role in the organization of the LPS monolayer in the outer membrane. This is seen both in isolated LPS and in the outer membrane of intact cells. The isolated LPS is known to contain inorganic (Na^+, K^+, Mg^{2+}, and Ca^{2+}) and organic (the polyamines putrescine, spermine, and spermidine, as well as ethanolamine) cations (49, 76). Galanos and Lüderitz (76) showed that the nature of cations had a decisive influence on the physical aggregation state of LPS in water, by removal of most of the cations by electrodialysis and reintroduction of known cations. Thus Ca^{2+} salts of LPS were essentially insoluble as expected from the bridging effect of divalent cations and from the dehydrating action of Ca^{2+} ion. The aggregate sizes were small and LPS was very water soluble when monofunctional amines were used for neutralization; probably the diffuse distribution of charges in amines and the bulky size of the compounds made tight association of LPS molecules difficult. The dissociating activity of triethylamine, which was the strongest of the compounds tested, seems to confirm this idea. The effects of Na^+ and K^+ were in between those of Ca^{2+} and organic amines. Furthermore, some anionic groups in the LPS are apparently arranged so that they accommodate divalent cations with very strong affinity, as was indeed shown by Schindler and Osborn (261).

There are many studies that show the importance of cations in the organization of outer membrane in bacterial cells. Among the most convincing are those which show that the outer membrane can be disorganized by removing divalent cations with chelators, as discussed later. On the other hand, addition of Mg^{2+} seems to stabilize the outer membrane. Thus, 0.1 to 5 mM Mg^{2+} in the medium was found to inhibit the leakage of periplasmic enzymes from deep rough LPS mutants (36) and from lipoprotein-deficient mutants (205). The sensitivity of deep rough mutants to hydrophobic inhibitors is reported to be decreased by Mg^{2+} (281), as is also the bactericidal effect of normal serum on smooth and rough enteric bacteria (182, 243).

Very high concentrations of divalent cations, especially Ca^{2+}, in the cold seem to disrupt the outer membrane. Thus treatment with 20 mM or higher concentrations of Ca^{2+} can make *E. coli* and *S. typhimurium* act as recipients in transfection (163) and transformation (43, 134). Under these conditions, the MBP in the periplasm becomes accessible to antibodies and higher-molecular-weight inhibitors such as amylopectin (27, 28). Furthermore, treatment with 100 mM or higher concentrations of Mg^{2+} in the cold releases significant amounts (35 to 52%) of the periplasmic β-lactamase from *S. typhimurium* (306). The most likely explanation seems to be that the extensive binding of divalent cations to LPS "freezes" the LPS monolayer by raising its melting

temperature enormously (as has been observed with other acidic lipids [228]) and at low temperature creates "cracks" in the outer membrane (27).

The interaction of polycations such as polymyxin, lysine polymers, and others with the anionic groups of the outer membrane is discussed below in connection with the barrier functions of the outer membrane.

Interaction Between Proteins and LPS

For the outer membrane to act as an efficient barrier, the interstices between the protein molecules and lipids should remain tightly sealed. One therefore expects the presence of a strong interaction between outer membrane proteins and lipids. The first indication of such an interaction between proteins and LPS came from the findings of Ames et al. (5) and Koplow and Goldfine (129) that levels of major outer membrane proteins were drastically decreased in *S. typhimurium* and *E. coli* mutants synthesizing very defective LPS, i.e., the deep rough mutants (see also Fig. 2), which became extremely sensitive to various hydrophobic agents as a consequence of this structural reorganization of the outer membrane (see below). Although the identity of *S. typhimurium* major outer membrane proteins had not been known at the time this work was published, the data show that the decrease was most pronounced in two porin species (OmpF and OmpD), with less pronounced effects on OmpC porin and the OmpA protein (see Fig. 4 of reference 5). It should be emphasized that in Ra, Rb, or Rc mutants (cf. Fig. 2) very little change was seen in the proteins levels and the decrease occurred suddenly when the nonreducing terminal glucose residue of the Rc LPS was lost by mutation (5, 278). Concomitantly with the decrease in proteins in deep rough mutants, the phospholipid content of the outer membrane showed a significant increase, whereas the LPS content stayed constant (278) or showed only a small (20 to 30%) increase (Goldberg and Nikaido, unpublished data).

With *E. coli* K-12, the outer membrane protein/LPS ratio was found to be decreased threefold in one heptoseless (i.e., Re-equivalent) strain (129). Lugtenberg et al. (150) reported a 30 to 45% decrease in the major outer membrane protein/total cellular protein ratio in similar strains and showed that the decrease was mainly due to the almost total disappearance of the OmpF porin. There have been conflicting reports on the outer membrane phospholipid/LPS ratio of *E. coli* mutants, but we do not see any reason that the situation with *E. coli* should be substantially different from that with *S. typhimurium* (for details, see reference 307a).

There are few pieces of evidence concerning the mechanism of lowering of protein levels in these mutants. However, from the beginning it was hypothesized that the assembly and organization of the outer membrane require specific interactions between proteins and LPS and that these interactions could not occur properly with the deep rough LPS with its very defective structure. What are the structural features of the LPS recognized by the proteins? They could include the presence or absence of the glucose residue linked to heptose (Fig. 2), but it is intriguing that the Rd_1 LPS of salmonella is reported to lack the phosphate group on the distal heptose residue (77). Similarly, the RcP^- mutant of *S. minnesota* (Fig. 2) is sensitive to hydrophobic agents (264,265). In *E. coli* K-12, strain D21e7 is sensitive to hydrophobic agents (see below) and its LPS lacks the phosphate groups on the heptose residue, although it contains the glucose residue linked to heptose (Fig. 2). These results all suggest that the presence of phosphate residues may be even more important than that of the glucose residue; a possible implication of these results is discussed in the following section.

When porins are isolated in detergent solutions, including SDS, they are known to contain significant amounts of LPS even after purification by gel filtration (see subsection, "Properties of Porins"). Furthermore, the heating of the porin/LPS complex at 100°C in SDS was found to dissociate the LPS (335). Since this treatment denatures the porin, the result suggests that a native conformation of the protein is necessary to adsorb LPS. As described above, Schindler and Rosenbusch (260) found that LPS-free porins inserted into planar bilayers of soybean phosphatidylcholine could not form stable channels, whose production was found to require both the presence of LPS and the subsequent aggregation of trimers.

Nakae (184) has shown that *S. typhimurium* and *E. coli* porins can be reconstituted with LPS and phospholipids to produce vesicles permeable to low-molecular-weight solutes. In these experiments LPS was necessary, but the reconstitution medium contained Mg^{2+}, and under these conditions vesicles could not be formed without LPS. Thus it is impossible to assess, from these data, the role of LPS in the promotion of channel formation by porins. In more recent studies, porins and LamB proteins have been reconstituted with phospholipids alone, even with heterologous phospholipids such as egg phosphatidylcholine, and were shown to exhibit full channel activity (144, 204, 208, 209). However, in this system the mass flux of water may be contributing to the stable opening of the channel.

The *E. coli* OmpF and OmpC porins act as receptors for the phages; the roles LPS plays in this function have been described above.

The specific interaction between an outer membrane protein and LPS has been most decisively shown for the OmpA protein. Several lines of evidence suggest that the OmpA protein is the specific receptor for phage TuII* in vivo (52). However, the isolated OmpA protein, dissolved in Triton X-100–bicarbonate buffer, did not inactivate this phage (52, 271). The inactivation occurred when LPS or lipid A of *E. coli* K-12 was added to the OmpA protein and was maximal when the LPS/OmpA mixture was precipitated by Mg^{2+} or phospholipids. Isolated core oligosaccharide inhibited the action of lipid A (271). Consistent with these results, dispersions made from OmpA protein and LPS, but not from OmpA protein and phospholipids, acted as phage receptors. Comparable results were obtained for phage K3, another phage that uses the *E. coli* OmpA protein as receptor (317).

OmpA protein appears to be needed in F-plasmid-mediated conjugation to stabilize the mating aggregate (164, 277). However, purified OmpA protein inhibits conjugation only when it is added together with LPS (1, 270, 316).

When the OmpA protein of *E. coli* is heated to 100°C in the presence of SDS, its apparent molecular weight upon SDS-polyacrylamide gel electrophoresis increases from 28,000 to 33,000, as described earlier. Addition of LPS to the heated OmpA protein has been described to cause its renaturation into the more compact, faster-migrating form (271). (However, LPS is not absolutely necessary for this process, as similar renaturation occurs also in the presence of 0.3 M NaCl [168].) LPS also protected the isolated OmpA protein from proteolytic cleavage to exactly the same extent as it did in the cell envelope, i.e., allowing the cleavage at the hinge region but preventing the further degradation of the part presumably buried in the LPS bilayer (271).

Beher et al. (21) have devised a method to measure the binding of OmpA protein to LPS by the use of LPS-coated erythrocytes, and they showed that the OmpA protein from K-12 bound effectively to K-12 LPS but not to LPS from unrelated clinical isolates. The OmpA proteins from the clinical isolates in turn adsorbed well to the homologous LPS preparations. Furthermore, when the K-12 *ompA* gene was transduced into the clinical isolates, it was very poorly expressed. However, mutants with better expression could be selected, and these mutants were found to produce an altered OmpA protein, which interacted much better with the endogenous LPS in the in vitro assay. On the other hand, Cole et al. (44) found that the *ompA* genes from *Shigella dysenteriae*, *Enterobacter aerogenes*, and *Serratia marcescens* were fully expressed in *E. coli* K-12. This was unexpected because some of these species are rather distantly related to *E. coli* and are expected to produce LPS different from that of K-12. The reason for this discrepancy is not known.

Mutants of *S. typhimurium* possessing a temperature-sensitive 3-deoxy-D-*manno*-octulosonate-8-phosphate synthetase (*kdsA* mutants) are unable to synthesize a complete lipid A under nonpermissive conditions (42°C). Recently, Rick et al. (247) showed that, when this mutant is shifted to 42°C, the rate of OmpA protein synthesis was not decreased but was markedly (2.5-fold) increased. There is no simple explanation for this surprising observation.

Barrier Properties of the Assymmetric Bilayer

Phospholipid bilayers are known to be permeable to hydrophobic molecules, and the permeability is higher for more hydrophobic solutes (for example, see reference 42). In contrast, the outer membrane of enteric bacteria does not appear to show a high degree of permeability to hydrophobic molecules, as shown by the strong resistance of these bacteria to hydrophobic antibiotics (e.g., macrolides, novobiocin, rifamycins, actinomycin D), detergents (e.g., SDS, bile salts, Triton X-100), and hydrophobic dyes (such as the ones used in the selective media for these bacteria, e.g., eosine, methylene blue, brilliant green) (136, 207). In fact, the very low permeability of outer membranes of the wild type (S-form) and Ra through Rc mutants (cf. Fig. 2) of *S. typhimurium* to a hydrophobic penicillin, nafcillin, has been demonstrated by directly measuring its rate of diffusion into the cell interior (200). Since many of these molecules are too large or too hydrophobic to permeate through the porin channel, the results indicate the unusually low permeability of the lipid bilayer region.

Loss of the next few sugar residues to produce the Rd and Re chemotypes (Fig. 2), however, makes the mutants extremely sensitive to the hydrophobic agents mentioned above (249, 257, 264–266). Consistent with these results, the diffusion rate of nafcillin through the outer membrane becomes suddenly elevated by a factor of 30 to 100, when going from Rc to Rd_1 mutant (200) (Fig. 7). Furthermore, the uptake of gentian violet, a hydrophobic bulky dye, was low (20%) in strains with S to Rc LPS, intermediate (45%) in strains with Rd_1 LPS, and high (above 60%) in strains with Rd_2 LPS (281).

Increased permeability to hydrophobic agents has also been demonstrated in *E. coli* K-12 mutants lacking heptose or having a somewhat less severe defect in LPS, involving the lack of galactose and glucose (296, 297). Further studies revealed that the loss of two of the three glucose residues and the galactose residue (*E. coli* K-12 strain D21e7; for

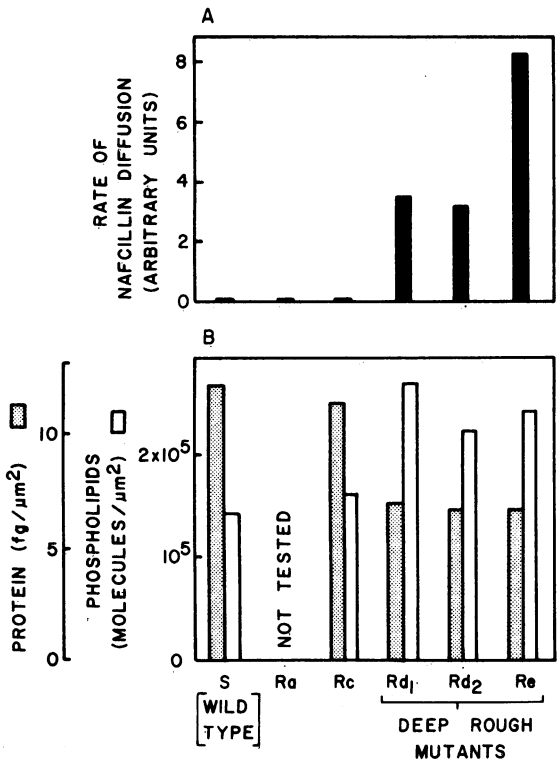

FIG. 7. Protein and phospholipid content per unit area of outer membrane in the wild type as well as mutant strains of *S. typhimurium* (A), and rate of nafcillin diffusion across the outer membrane (B). LPS content per unit area was reported to be constant (278), but recent reexaminations show a slight (20 to 30%) increase in Rd and Re mutants (Goldberg and Nikaido, unpublished data). Taken from reference 202 with permission from J. Wiley & Sons, Inc.

K-12 LPS structure, see Fig. 2) was already sufficient to increase the permeability (91). So-called *nbsB* mutants of *E. coli*, sensitive to a number of hydrophobic agents, were shown to produce LPS molecules variably deficient in their deep core portion, due to a leaky *rfaD* mutation (45, 46). Deep rough mutants of *Proteus mirabilis* were also found to be hypersensitive to various hydrophobic agents (256), and a rough mutant of *P. aeruginosa*, hypersensitive to penicillin G and some detergents, but not to most dyes, has been reported (131).

Other hydrophobic agents to which the deep rough mutants become sensitive include fatty acids (273), phenol (266), polycyclic hydrocarbons (4), more hydrophobic derivatives of tetracycline (137), and anionic (257, 266, 284) as well as cationic (306) detergents. Deep rough mutants show only a slightly increased sensitivity to the polycationic antibiotic polymyxin (249), which is not particularly hydrophobic, and has an affinity to LPS (10, 177, 261; see also below).

In contrast to the increased sensitivity to hydrophobic antibiotics, the deep rough mutants do not show hypersensitivity to hydrophilic agents. In some cases even a decrease of sensitivity is found, for example, for penicillin, ampicillin, cephalothin, and tetracycline (249, 257, 264). This is presumably due to the decrease in the number of porins in the outer membrane (see above); since these relatively bulky and slightly hydrophobic compounds are barely able to go through the porin channel, their permeability is affected strongly by the number and the nature of porin channels present.

Several hypotheses can be proposed to explain the increased permeability of the outer membrane of deep rough mutants to hydrophobic agents. (i) The carbohydrate chain of LPS may produce a surface layer that acts as a barrier against the diffusion of hydrophobic molecules. This layer is essentially absent in deep rough mutants. (ii) Deep rough mutants contain phospholipid molecules in the outer leaflet, and the phospholipid bilayer domains thus created make a major contribution to the increased permeability (Fig. 6B). (iii) A strong LPS-LPS interaction is essential in preventing penetration by hydrophobic solutes in the wild type (Fig. 6A). Such a strong lateral interaction is lacking in the altered, deep rough LPS. Thus hydrophobic solutes penetrate through the LPS monolayer domains easily (Fig. 6C), regardless of the presence or absence of phospholipid bilayer domains.

We rejected hypothesis (i), mainly because Rc mutants are not hyperpermeable despite the absence of 80 to 90% of the saccharide chain found in the wild-type LPS, whereas the loss of only one additional glucose residue led to a sudden increase in permeability (201, 207). This hypothesis, however, was recently resurrected by Hiruma et al. (110). We do not believe that this is the correct interpretation of their results. We emphasize here that even the most concentrated, densely intertwined polysaccharide matrix produces only a 3% reduction in the rate of diffusion of small solutes (62).

In deep rough mutants both the phospholipid content (and location) and the structure of LPS are altered at the same time; therefore, it is difficult to know which one is more responsible for the observed change in permeability, i.e., to choose between hypotheses (ii) and (iii). There were mutants (*omp-1010* and *omp-1012*) of *S. typhimurium* which incorporated reduced amounts of outer membrane proteins (5), produced LPS that appeared normal in its composition, including the content of free NH_2 group and heptose phosphate (5), and yet had phospholipid bilayer domains in the outer membrane (121) and were hypersensitive to hydrophobic agents (5). This observation obviously favored hypothesis (ii). In addition, hypothesis (ii) is based on the observed facts and requires no additional assumptions. It can also explain other phenomena, such as the increased permeability of EDTA-treated cells (Fig. 6D). These are the reasons why we have favored hypothesis (ii) over (iii) during the last several years (201, 202, 207).

We cannot, however, rule out hypothesis (iii) at present. In fact, there are pieces of evidence that slightly favor this idea. First, although the existence of phospholipid domains in the outer membrane of deep rough *S. typhimurium* mutants is well established (121), the fraction occupied by such domains may not be extensive. The amount of phospholipid in the outer membrane increases by 30 to 50% in the deep rough mutants (Fig. 7). Although we thought that LPS did not show any increase (278), more recent experiments showed a modest yet significant increase of 20 to 30% (Goldberg and Nikaido, unpublished data). This leaves only a small area, perhaps less than 10% of the outer membrane, as the phospholipid bilayer; indeed, the fraction of phosphatidylethanolamine head groups accessible to an impermeable reagent in the deep rough outer membrane was only about 8% of the total, after the correction of the labeling efficiency (121). Second, as described earlier, the hypersensitivity in various species seems to correlate well with the loss of one of the phosphate groups in LPS, rather than with the loss of any particular sugar residue. Because bridging of negatively charged phosphate groups appears to be very important in LPS-LPS interactions, it is conceivable that deep rough LPS is significantly deficient in this respect. Third, alteration of LPS structure seems to change the permeability of the outer membrane without involving the loss of much proteins, as seen in the *acrA* mutants (see below). Finally, Parton (229) found that deep rough mutants of *S. minnesota*, which were hypersensitive to hydrophobic agents, did not show a striking decrease in the major outer membrane proteins, although a quantitative analysis has not been performed.

We have to conclude, therefore, that we do not yet have a final answer on the molecular basis of increased permeability in deep rough mutants. Studies using reconstituted systems are sorely needed to settle this question, and the liposome study of Hiruma et al. (110) is a step in the right direction, although we cannot agree with their interpretation of data. However, regardless of the final answer, it is clear that the LPS outer leaflet of wild-type bacteria, with its tight LPS-LPS interactions, is serving as a very effective barrier against the penetration of hydrophobic molecules.

We emphasize here that the low permeability of the outer membrane lipid bilayer to hydrophobic solutes does not mean that the permeability has an inverse correlation with hydrophobicity. The permeation is assumed to occur through the intermediate stage of the dissolution of the permeant into the hydrophobic interior of the membrane, and such a process dictates that the more hydrophobic solutes would penetrate more rapidly. It seems reasonable that the permeability-versus-hydrophobicity curve for the outer membrane is displaced to the more hydrophobic side in comparison with the usual phospholipid bilayer membranes (Fig. 8). This notion is consistent with the observation that gram-negative bacteria do not seem to have much problem with the uptake of O_2 and that some of them utilize alkanes as the carbon source. It was also shown that a very hydrophobic tetracycline derivative, minocycline, which has an apparent partition coefficient of 25 even in its ionized form (137), crosses *E. coli* outer membrane at a rate faster than that of tetracycline (169), which diffuses through the porin channel rather slowly owing to its large size and negative charge. (However, the conclusion that minocycline crosses the outer membrane 10 times faster than tetracycline [169] is misleading because the driving force for influx across the outer membrane was much greater for minocycline than for tetracycline. Calculations [Nikaido, unpublished data] suggest that there is likely to be a less than twofold difference in the outer membrane permeabilities of the two compounds.)

Deep rough mutants of *S. typhimurium* release periplasmic enzymes into the medium (36, 138). The most likely explanation of this phenomenon is the transient rupture and resealing of the outer membrane (207). Deep rough mutants also become more sensitive to lysozyme (257, 296); possibly this is caused by the same instability of the outer membrane (see also below).

Some nonenteric bacteria including *N. gonorrhoeae* are quite sensitive to hydrophobic antibiotics and dyes. At first it seems possible that in these bacteria the outer membrane contains phospholipid bilayer regions even in wild-type cells. However, in *N. gonorrhoeae*, a mutation at the *env* locus makes the cells even more sensitive to hydrophobic agents such as erythromycin, rifampin, fusidic acid, Triton X-100, and crystal violet (258), and this is accompanied by an increase in the phospholipid/protein ratio (155), as well as an increased susceptibility of outer membrane phospholipids to attack by phospholipase C (155). If the outer membrane of the wild type already contained large areas of phospholipid

bilayer, it may be difficult to increase the permeability substantially by further increases in the phospholipid bilayer domains. We should not, therefore, rule out the possibility that the bilayer in the wild type is totally asymmetric as in the enteric organisms, but the weaker LPS-LPS interaction in this organism allows the penetration of hydrophobic molecules. Another mutation, *mtr*, produces decreases in sensitivity to hydrophobic agents (157), accompanied by production of a 52,000-dalton outer membrane protein (92). About one-quarter of *N. gonorrhoeae* isolates from the rectum of male homosexual patients contained *mtr* mutations, making them more resistant to fecal lipids (178). Another mutation, *penB*, increases resistance to penicillin and tetracycline further when it exists together with *mtr*. Although *penB* strains were initially thought to have an altered "principal outer membrane protein" (92), i.e., the porin (60), a more recent study showed that this was due to the cotransformation of *penB* with the nearby *nmp-1* gene (32).

Effects of EDTA

Twenty-six years ago, Repaske (241) showed that EDTA and Tris buffer are necessary, in addition to lysozyme, to convert gram-negative bacterial cells to spheroplasts, and we now recognize the role of EDTA as that of weakening the LPS-LPS interaction and disrupting the outer membrane.

Leive (135) showed that EDTA, again in the presence of Tris buffer, released about one-half of LPS but little else from *E. coli* cells and made the cells sensitive to a number of hydrophobic compounds, including actinomycin D, novobiocin, etc. (for a comprehensive list see reference 136). (A similar release of LPS was also found in *P. aeruginosa* [89].) Because the space that had been occupied by the released LPS must become occupied by other hydrophobic components, Nikaido and Nakae (207) proposed that this space would be filled by phospholipids (Fig. 6d), either from the inner leaflet of the outer membrane or from the cytoplasmic membrane. The presence of phospholipid molecules in the outer leaflet will create phospholipid bilayer regions just like in the outer membrane of deep rough mutants, and this altered organization of the outer membrane will explain the permeability properties of the outer membrane of EDTA-treated cells. The rapid reorganization of the lipid components of the peptidoglycan-free outer membrane (179) and the reversible flow of phospholipids between outer and inner membranes (120) are consistent with this hypothesis, which, however, remains to be tested in a critical manner. An alternative explanation is that the LPS-LPS interaction is weakened by the removal of divalent cations and that the LPS monolayer allows the penetration by hydrophobic molecules, a situation similar to that shown in Fig. 6C. This is less attractive, however, because it cannot explain the need for active biosynthesis (136) for the reestablishment of the effective barrier. In electron micrographs, the outer membrane of EDTA-treated *E. coli* was more easily fractured by freeze-fracturing, and the surface pits were less clearly observed (17); these are both characteristics of the deep rough outer membrane. Interestingly, EDTA treatment of wild-type *E. coli* cells resulted in the production of free fatty acids, presumably owing to the activation of endogenous phospholipases (97). When a mutant defective in these enzymes was treated with EDTA, as much LPS was released from it as from the wild-type strain, but a lesser degree of sensitization to actinomycin D was observed (97). Thus, hydrolysis of phospholipids may enhance the reorganization of the outer membrane. Other enterobacterial mutants resistant to Tris-EDTA-induced permeability increase are the polymyxin-resistant *pmrA* strains of *S. typhimurium*, which have an altered LPS (see below), and the less well-characterized strains of *E. coli*, studied by Voll and Leive (325), and *Shigella flexneri*, studied by Corwin et al. (48). All of these mutants release less LPS upon EDTA treatment than do the corresponding wild-type strains.

The rapid dilution of *E. coli* cells preincubated in 20% sucrose containing EDTA and Tris buffer ("the osmotic shock procedure") results in the release of periplasmic enzymes (194); again Tris is needed and at high concentrations could even replace EDTA. We now understand the mechanism of this treatment. During preincubation, the periplasmic space becomes filled with 20% sucrose, which produces plasmolysis. Upon dilution, the system will try to correct the osmotic imbalance between the medium and the periplasm through the efflux of sucrose through the porin channel and the influx of water. Because the former is a slow process due to the large size of sucrose, the outer membrane becomes ruptured by the more rapid influx of water into periplasm.

Clearly, in all the above cases, the removal of divalent cations is increasing the electrostatic repulsion between neighboring LPS molecules or between LPS and acidic proteins. Tris, a bulky primary amine, would further contribute to the destabilization by partially replacing other cations bound to LPS (see also reference 261); we have seen that organic monofunctional amines discourage tight LPS-

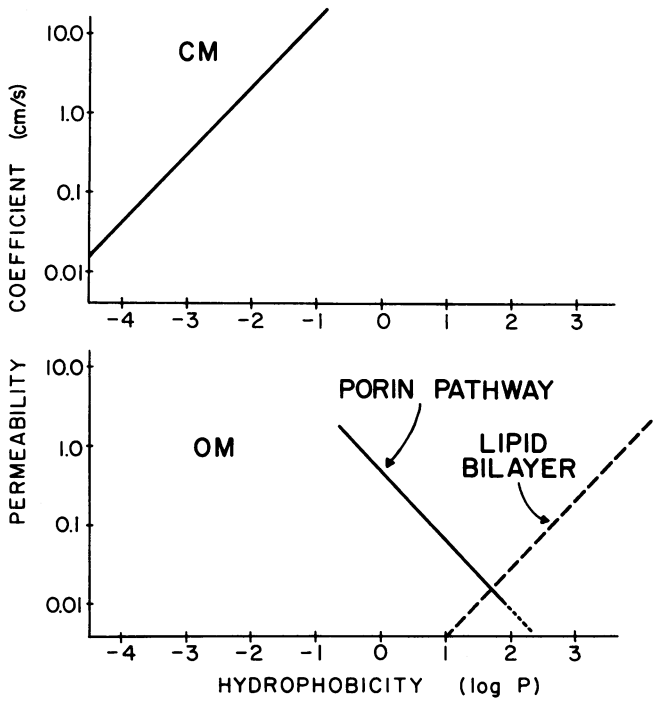

FIG. 8. Predicted penetration rates of compounds of different hydrophobicity across outer and inner membranes by simple diffusion processes. Note that in the outer membrane (bottom) hydrophilic compounds can penetrate through the porin channels, which do not exist in the inner membrane (top). In contrast, in the outer membrane the curve for the diffusion of hydrophobic compounds through the lipid interior is displaced to the right, presumably because of the high resistance offered by the LPS monolayer. The permeability coefficient values shown are entirely hypothetical.

LPS interactions (see above). It should also be noted that high concentrations of Tris alone (0.1 M, pH 7.2), without EDTA, are sufficient to release significant amounts (20%) of LPS from smooth *S. typhimurium* (R. Hukari and M. Vaara, unpublished data). Tris also sensitizes smooth bacteria to complement (243) and renders the outer membrane of an Rc mutant of *S. typhimurium* susceptible to labeling with dansyl chloride (263). After treatment of *E. coli* O8 with Tris, the periplasmic alkaline phosphatase appeared to move into the outer membrane, thus becoming more accessible for *p*-nitrophenyl phosphate in the medium (119).

It is thus far unknown why Tris-EDTA is able to liberate maximally only approximately half of the LPS from the outer membrane. This might even suggest the existence of the LPS in two different domains in the outer membrane (136). Nevertheless, the LPS population released by EDTA from smooth bacteria (*S. typhimurium*) shows an O-chain length distribution identical to that of the unreleasable LPS (Hukari and Vaara, unpublished data).

Effect of Polycations

Polymyxin is a polycationic decapeptide antibiotic with a fatty acid "tail" and with five positively charged groups and no negatively charged group. Its ultimate lethal target is generally thought to be the cytoplasmic membrane, to which it will become bound through its affinity to phospholipids (300), thereby destroying the barrier property of the cytoplasmic membrane (285). However, for this binding to happen, polymyxin molecules must cross the outer membrane barrier. Polymyxin B is too large to go through the narrow porin channel of the enteric bacteria, and therefore it is thought to gain access to the cytoplasmic membrane through the disruption or disorganization of the outer membrane. Indeed, polymyxin binds to the outer membrane (300), causes extensive, electron microscopically visible alterations there (142, 262), and is known to disrupt the outer membrane permeability barrier to many agents such as hydrophobic antibiotics (253), detergents (308), and lysozyme (299).

Further evidence that the disorganization of the outer membrane is the first step in the action of polymyxin was obtained through the study of polymxyin-resistant *pmrA* mutants of *S. typhimurium*, isolated by Mäkelä et al. (160). These mutants have a low-level (100 μg/ml) resistance to polymyxin. Their outer membrane (in contrast to the outer membrane of the parent strains) did not become permeable to lysozyme or the anionic detergent deoxycholate upon treatment with polymyxin (308). This indicates that the *pmrA* mutation affects the outer membrane and decreases its susceptibility to polymyxin. The mutant cells and the LPS isolated from them bound less polymyxin than the parent wild-type strains and their LPS, respectively (312, 313). The LPS of the *pmrA* mutants was shown to contain four- to sixfold larger amounts of 4-aminoarabinose and also larger amounts of ethanolamine than the wild type LPS, making the mutant LPS less acidic or decreasing its net negative charge (312). This can obviously explain the decreased binding of polymyxin B by the mutant LPS. (Hancock and co-workers [92a, 95a] recently proposed that other polycationic antibiotics, notably aminoglycosides, also cross the outer membrane barrier of *P. aeruginosa* by using a mechanism similar to that used by polymyxin B, i.e., by first binding to LPS and thereby disorganizing the outer membrane.)

The *pmrA* mutants were also found to be more resistant to the action of Tris-EDTA as well as polycations such as polylysine and protamine (306). These results are again consistent with the decreased net negative charge on the LPS. However, the sensitivity of the *pmrA* mutants to octapeptin (a compound with a structure similar to polymyxin except that it is an octapeptide, rather than a decapeptide, and has a fatty acid tail two methylene units longer) and cationic detergents such as benzalkonium chloride and cetyltrimethylammonium chloride was not reduced; perhaps the hydrophobic interaction plays a more predominant role in the disorganization of the outer membrane by these agents.

More recently, Vaara and Vaara (309–311) studied the interaction of gram-negative bacteria with hydrophilic polycationic agents. These included oligomers of lysine, as well as the papain-cleaved derivative of polymyxin that has lost the N-terminal diaminobutyric acid residue with the attached fatty acid residue (polymyxin B nonapeptide [PMBN]). Both PMBN and Lys_{20} had no or very low antibacterial activity against wild-type strains of *S. typhimurium* and *E. coli*, but were remarkably active in sensitizing them to hydrophobic agents such as novobiocin, fusidic acid, erythromycin, clindamycin, rifampin, actinomycin D, cloxacillin, and nafcillin. Sensitivity comparable to that of the deep rough (Re; Fig. 2) mutant was obtained by the addition of <1 to 10% of the MICs of these polycations (309, 310). PMBN was also shown to sensitize *E. coli* to the hydrophobic ionophores valinomycin and A23187 (3), whereas a very slight or no increase in sensitivity was found to agents such as benzylpenicillin, ampicillin, and tetracyclines, which are believed to penetrate the outer membrane through porin pores (325). Large lysine polymers (Lys_{50}) and protamine were bactericidal but, at sublethal concentrations, sensitized the bacteria to hydrophobic antibiotics, whereas Lys_4, streptomycin, cytochrome *c*, lysozyme, and the polyamines cadaverine, spermidine, and spermine had neither bactericidal nor sensitizing activity.

Further studies revealed that Lys_{20} and PMBN apparently acted through different mechanisms. Lys_{20} caused the release of about 30% of LPS from the cell, and the sensitization to hydrophobic agents apparently required a long lag. In contrast, PMBN did not release any LPS from the cells, and the sensitizing action was very rapid (311). PMBN was shown to produce long, finger-like projections involving only the outer leaflet of the outer membrane, whereas the main effect of Lys_{20} was merely to increase the wrinkled appearance of the outer membrane in this section (309). At present it seems that Lys_{20} might act in a way rather similar to EDTA by removing LPS and creating phospholipid bilayer regions in the outer membrane. In contrast, PMBN, with its precisely positioned positive charges, first seems to bind to the LPS and expand the areas occupied by the outer leaflet significantly, leading both to increased permeability and to the creation of finger-shaped protrusions.

Besides wild-type strains of *E. coli* and *S. typhimurium*, the strains susceptible to the PMBN-induced sensitization to hydrophobic antibiotics included *P. aeruginosa*, *P. maltophilia*, *Acinetobacter calcoaceticus*, *Klebsiella pneumoniae*, *K. oxytoca*, *Enterobacter agglomerans*, and *Enterobacter cloacae* (324), as well as an *E. coli* strain lacking the outer membrane-associated phospholipase (314). Accordingly, this enzyme is not the mediator in the increase of permeability. However, the polymyxin-resistant gram-negative bacteria (*Proteus* sp. strains and *Providencia stuartii*, *Serratia marcescens*, and the *pmrA* mutant of *S. typhimurium*) were

resistant to PMBN and Lys_{20}, a result indicating that these agents bind to the same target (acidic LPS) as polymyxin in the outer membrane (307, 324). By using tritium-labeled PMBN, it has been shown that polymyxin and PMBN compete with each other for their binding to the outer membrane. Approximately 10^6 molecules of PMBN are bound per single cell of *S. typhimurium* and the binding is of relatively high affinity, with a K_d of 1.3 μM (M. Vaara and P. Viljanen, submitted for publication). PMBN was also shown to sensitize serum-resistant enteric bacteria to lethal membrane attack by the complement cascade (309). The bactericidal system also required an absorbable factor, present in normal sera (315). This factor is presumably antibodies against the O-serological part of the LPS because antibodies against other cell envelope components are inactive in restoring the bactericidal system (H. Käyhty, M. Vaara, and P. H. Mäkelä, submitted for publication). It is hoped that further biochemical analysis of the actions of polycations on the outer membrane may shed light on the details of the molecular organization of the outer membrane.

Low-level polymyxin-resistant mutants of *P. aeruginosa* were found to be unaltered in porin, but to overproduce another protein, H1, of 21,000 apparent molecular weight (196). This protein was overproduced also if the wild-type strain was starved for Mg^{2+}, a treatment that slightly (from 1 to 10 μg/ml) increased the resistance of the organism to polymyxin B. The mutant, as well as the wild-type cells grown under Mg^{2+} starvation, is resistant to EDTA. These results are reminiscent of the properties of the *pmrA* mutants of *S. typhimurium*, and Nicas and Hancock (196, 197) suggest that protein H1 substitutes for Mg^{2+} that is bound to LPS, thereby preventing the binding of polymyxin. Determination of Mg^{2+} levels showed that there was a moderate decrease (down to about 60% of the wild-type level) in the H1-overproducing mutant.

The results described above suggest that different bacteria may use different strategies to develop resistance to polymyxin. Since the porin channels of *P. aeruginosa* are larger (94, 339), in principle there should be a possibility for polymyxin to reach the target by diffusing through these channels. However, the contribution of this pathway may be minimal, as most of these channels seem to be "closed" (22, 338). This prediction is borne out by the observation of Nicas and Hancock (196) that a *P. aeruginosa* mutant deficient in porin showed an unaltered sensitivity to polymyxin. Gilleland and Lyle (83) showed that high-level (950 to 2,000 μg/ml) polymyxin-resistant strains of *P. aeruginosa* appeared to be deficient in porin, in addition to other alterations. This is consistent with the idea that the *P. aeruginosa* porin channel may catalyze the slow, residual diffusion of polymyxin, although the LPS alteration of *pmrA* mutants in enteric bacteria does not produce such high-level resistance in spite of the expected inability of the narrow enteric pores to catalyze the diffusion of the agent.

There is growing evidence that certain cationic host defense factors increase the outer membrane permeability. One such agent, protamine, having a high arginine content, has long been known to be inhibitory to gram-negative bacteria and at sublethal concentrations has recently been shown to increase the outer membrane permeability to sodium deoxycholate and novobiocin (306). Another is the "bactericidal permeability-increasing protein," or BPI, of Elsbach and Weiss (64), isolated from the granule contents of polymorphonuclear leukocytes. It is strongly basic (pI > 9.6), complexes with isolated LPS, binds to the outer membrane, and increases its permeability to hydrophobic antibiotics. Furthermore, *Proteus* sp. and *Serratia* sp. strains (known to be polymyxin resistant) are resistant to the actions of BPI. Interestingly, this basic protein was relatively inactive on smooth, wild-type strains of *S. typhimurium* and *E. coli* presumably because its access to the inner core portion of LPS was hindered by the O-antigenic polysaccharide (330). In contrast, smaller PMBN molecules were fully active against smooth strains of these organisms (see above).

Potential Dynamic Fluctuations in Structure

When the permeability of outer membrane to hydrophobic compounds was measured in intact cells, it was observed that the use of freshly harvested cells was essential (200). When the washed cells were left at room temperature for more than 30 min, they became highly permeable to various hydrophobic compounds. Helgerson and Cramer (103) found that *E. coli* cells treated with an uncoupler, carbonyl cyanide-*m*-chlorophenylhydrazone, showed higher fluorescence with the exogenously added hydrophobic probes such as *N*-phenyl-1-naphthylamine and 8-anilino-1-naphthalenesulfonic acid and that deenergized cells as well as EDTA-treated cells bound more *N*-phenyl-1-naphthylamine. From these results they suggested that deenergization lowers the permeability of the outer membrane. Tecoma and Wu (298) similarly found that deenergization of *E. coli* by colicin K dramatically increased the fluorescence caused by the exogenously added polyene fatty acid probe *cis*-parinaric acid. This was shown clearly to be the result of an increased binding, rather than of altered microviscosity of the inner membrane, as there was no effect of colicin K on fluorescence caused by parinanic acid residues incorporated into membrane phospholipids. Wolf and Konisky (332) found that larger amounts of azidopyrene, a highly hydrophobic, photolabile probe, were bound to *E. coli* cells when the cells were deenergized and the potential across the cytoplasmic membrane was diminished. Comparison with the labeling pattern of deep rough mutants suggested that significant changes in the structure and barrier properties of the outer membrane were induced by the deenergization of the inner membrane (333).

The mechanism involved in the transfer of information from the inner to the outer membrane is unknown. However, we note that, in *E. coli* cells suspended in a dilute buffer or medium, there is a Donnan potential (inside negative) across the outer membrane. There is, therefore, a proton gradient (periplasmic side high) across the outer membrane (283). This high proton concentration in the periplasm may in turn be balanced by the proton gradient (periplasmic side high) created by the electron transport process. When the gradient across the inner membrane is collapsed, there will be a continuous influx of H^+ through the outer membrane, which may result in a transient decrease in the Donnan potential.

The nature of alteration in the structure of the outer membrane is unknown. If the speculation presented above is correct, the conformation of some proteins might become altered due to the collapse of the Donnan potential, and this may open up pathways of leakage in the space between the proteins and the lipid components of the membrane. An alternative possibility involves the transient formation of non-bilayer lipids. In recent years ^{31}P-nuclear magnetic resonance studies showed that many biological membranes produce signals that can be interpreted as those of isotropic lipids, assumed to correspond to small inverted micelles and

to short hexagonal cylinders. It has been hypothesized that the transient occurrence of these non-bilayer structures is important in various dynamic functions of the membrane (50). One is tempted to speculate that this phenomenon may be involved in the transient increase in outer membrane permeability described above. However, ^{31}P-nuclear magnetic resonance studies of *E. coli* outer membrane showed the nearly complete absence of non-bilayer lipids in this structure (31).

Other Genes and Mutations Affecting Permeability

Mutants lacking one or more of the porins, or the OmpA protein, appear to have a nearly unaltered sensitivity to hydrophobic antibiotics, dyes, and detergents (53, 105, 216, 272, 277, 280). This is not too surprising because there is usually a compensatory increase in the amounts of major proteins left, so that the total protein content of the outer membrane is almost unchanged in these mutants (34, 143, 319). In this connection, an interesting observation was made by Siden and Boman (274). They isolated 25 *ompC* mutants of *E. coli* K-12 by phage selection and found that about one-half of them were hypersensitive to cecropin D, a 36-residue straight-chain antimicrobial peptide in the hemolymph of a *Cecropia* moth, as well as to a number of hydrophobic inhibitors and antibiotics. Since these mutants rapidly acquired secondary mutations that made them less sensitive to these agents, it is tempting to imagine that the loss of the OmpC porin created phospholipid bilayer patches such as in the deep rough mutants, but the phenomenon is often overlooked owing to the rapid accumulation of secondary mutations. An alternative possibility, however, is a true pleiotropic effect through the small RNA (micRNA) whose production is regulated by the transcription of the *ompC* gene (173).

A mutant missing both the OmpA protein and the porins was found to be more sensitive to EDTA and to deoxycholate (272) but showed an otherwise unchanged sensitivity pattern to hydrophobic agents (105, 272). This is unexpected, because these mutants are reported to have a lowered protein content and about twofold-increased phospholipid content in the outer membrane (272, 319, 320). One possible explanation is the increase in LPS content, which is reported to be twofold at least in one report (320).

In *S. typhimurium*, some of the genes needed for the biosynthesis of a component of ECA, 4-acetamido-4,6-dideoxy-D-galactose, are present in the *rfb* operon, which is mainly responsible for the biosynthesis of the O-antigen portion of LPS (H. Lew, Ph.D. thesis, University of California, Berkeley, 1978). The deletion of these genes makes the mutant cells hypersensitive to SDS, but not to erythromycin or a cationic dye, crystal violet (161). The deletion is believed to cause the accumulation of the carrier-linked intermediates of ECA synthesis, but the connection between this phenomenon and the SDS-hypersensitive phenotype is not clear.

Very recently, three new types of *S. typhimurium* mutants with increased sensitivity to hydrophobic agents have been isolated (287). No obvious changes were found in LPS, phospholipids, or outer membrane proteins in any of them. Furthermore, their growth rates and cell morphology were unaltered. One of the mutations (class B) was located between 7 and 11 min on the chromosome and might therefore represent the *Salmonella* equivalent of the *acrA* mutation in *E. coli* (see below).

Acridine dyes have been used for many years in the "curing" of sex plasmids from F^+ strains and of R plasmids from R^+ strains of *E. coli*. Although it is often assumed that this is caused by the interaction of these dyes with the supercoiled plasmid DNA, Yoshikawa (337) presented evidence that the male cells are preferentially killed by these dyes. This suggests that the presence of sex and R factors alters the permeability of the outer membrane. Indeed, this idea is supported by earlier observations that SDS, an anionic detergent, lyses the male, but not female, cells (2) and penicillin, a moderately hydrophobic agent (see above), was effective in curing the R factor from *S. paratyphi* (123). The mechanism for the presumed increase in bilayer permeability is unknown; possibly it involves the creation, accompanying the insertion of sex pili and other F- and R-factor-coded proteins, of loosely associated or somewhat disorganized protein/lipid interfaces. It is reported that strains carrying R plasmids in an integrated state are more sensitive to cholate (336).

The *acrA* mutation (at map position 10 min) of *E. coli* K-12 makes the strain more sensitive not only to cationic dyes such as crystal violet, methylene blue, and acriflavine, but also to phenethylalcohol and SDS (191), as well as to lincomycin and erythromycin (106). It was reported that these mutants produced LPS that totally lacked phosphate groups on the lipid A part (46), but this claim has since been retracted (137).

The mutation in the *envA* gene, located at the 2-min position of the *E. coli* chromosome in an area containing many genes involved in peptidoglycan synthesis and cell division, produces chains of cells and makes the cells susceptible to both hydrophobic (rifampin) and rather hydrophilic (β-lactams) agents (215). Apparently the structure of LPS is unaltered. Grundström et al. (90) showed that the LPS content of the outer membrane is decreased by about 25% in the mutant and that another mutation, *sefA1*, which increases the protein content of the outer membrane, suppressed the hypersensitivity caused by the *envA* mutation. The decreased level of LPS is likely to create phospholipid bilayer regions as described for the deep rough LPS mutants and explains the increased sensitivity to hydrophobic agents. However, the reason for the increased permeability for hydrophilic agents (about a fivefold increase in the diffusion rate of cephalosporin C was reported [90]) is not known (see, however, below).

Many of the colicin-tolerant mutants show alterations in sensitivity to detergents and hydrophobic antibiotics (53). For example, the *tolC* mutation resulting in the loss of a minor outer membrane protein (175) makes the cell extremely sensitive to deoxycholate, SDS, Triton X-100, phenethylalcohol, erythromycin, novobiocin, and fusidic acid. The outer membrane of *tolC* mutants was deficient in OmpF porin, a situation reminiscent of the cecropin D hypersensitivity of some *ompC* mutants observed by Siden and Boman (274) (see above). However, none of the "standard" *ompF* mutant strains is hypersensitive to detergents, as described earlier.

Richmond's group has isolated mutants of *E. coli* K-12 hypersensitive to a wide range of antibiotics for use in studying the permeability barrier of the wild-type organism (245). The mutants (DC2 and DC3) have been very useful in showing that the outer membrane is limiting the access of many antibiotics to the targets in the cytoplasmic membrane or in the cytoplasm, but they have also been misused by many investigators who believed that the mutants possessed no penetration barrier whatsoever. In fact, the mutants do

produce outer membrane, which does limit the diffusion of many solutes, depending on the circumstances (for discussion, see Nikaido, Pharmacol. Ther., in press). The mutants are hypersensitive to hydrophobic agents (the MIC of novobiocin is reduced 40-fold) and also to moderately hydrophilic agents (that of ampicillin reduced 16-fold). It has been reported that the mutant LPS has an altered amino group/phosphate ratio (40). The mutant DC2 appeared to contain physically and functionally unaltered porins (Nikaido, unpublished data).

A superficially similar mutant has been reported in *P. aeruginosa* (343). The mutation produces marked reductions in the MICs of β-lactams, and experiments showed clearly that the mutant outer membrane is much more permeable to these agents (343). The mutant LPS was found to differ from the wild-type LPS in the ratio of some core sugars and also in the content of dodecanoate and 2-hydroxydodecanoate (132, 343). Angus et al. (6) suggest that the altered LPS may increase the fraction of "open" channels when complexed with porin. However, Nikaido (unpublished data) could not detect any difference in permeability when the crude outer membranes from the wild type and this mutant (expected to contain a putative porin/LPS complexes) were reconstituted with phospholipids into proteoliposomes.

FUSION SITES BETWEEN OUTER AND INNER MEMBRANES

When Bayer (16) examined thin sections of *E. coli* cells plasmolyzed in 20% sucrose, he observed sites at which the inner membranes were apparently fused to the outer membrane. Since then, morphological studies revealed that they corresponded to sites of attachment and probably nucleic acid injection for a number of phages (16), as well as to sites of export of newly synthesized LPS (180) and porins (279) (for review, see reference 16). There have been speculations that the adhesion sites may also be involved in the uptake of various substances, especially macromolecules such as colicin (see reference 128) and DNA, but few data are available except in the area of nucleic acid injection by phage, mentioned above.

More recently, Bayer et al. (18) showed that fractions of buoyant density intermediate between inner and outer membranes of *S. anatum* contained structures in which small outer membrane vesicles were joined through a narrow junction to inner membrane vesicles. In cells infected with converting phage ε^{34} 6 to 8 min before harvest, the LPS with an altered structure, whose synthesis was directed by the ε^{34} genome, was seen in this intermediate density fraction but not yet in the outer membranes, showing that the intermediate density fraction contained regions of export of newly made LPS. The adhesion zone, however, constitutes only a small part of the structure dominated by the large vesicles of inner and outer membranes connected to it. Perhaps for this reason, demonstration of components specific to this zone has been difficult. Although phospholipase A was regarded by some as a marker enzyme for the adhesion zone, its specific activity in the intermediate density fraction is only slightly higher than that in the outer membrane (18). In this connection, it is interesting that penicillin-binding protein 3 was found only in the membrane fraction of intermediate density and not in the inner or outer membranes (250); we cannot exclude, however, the possibility that the protein belongs to a membrane fraction of another type that happens to share a similar density with the fusion sites.

MacAlister et al. (155a) recently showed, by electron microscopy of serial sections of *S. typhimurium* cells, that zones of adhesion on both sides of the cell division site are continuous and completely cover the circumference of the cells. These "periseptal annuli" produce a completely segregated periplasmic compartment at the site of septum formation, an observation that may have important implications on the mechanism of the latter process. However, we do not know at present the relationship, if any, between these circular adhesion zones and the pointlike adhesion sites observed earlier (16, 180, 279).

CONCLUSION

Bacterial outer membrane shows unusual functional properties, i.e., very low permeability toward lipophilic solutes and high permeability toward hydrophilic solutes. These functional attributes can be correlated well with the presence of unusual structural components, LPS and porins, and the precise molecular organization of these components. By using them, the gram-negative bacteria are able to produce a layer that acts as a very effective permeation barrier and at the same time still allows the efficient diffusion of nutrients.

Our current understanding of the molecular organization and functions of bacterial outer membrane was made possible by the availability of mutants and of conditions, such as EDTA or polycation treatment, that alter outer membrane permeability. Yet the properties of some of the mutants, as well as the alterations of permeability under certain conditions, suggest that we do not yet have a complete picture of the outer membrane structure. It is hoped that further studies will lead us to a better understanding of this topic.

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Some passages from a recent review (307a) by the same authors on a related topic have been used here without much alteration, with permission from Elsevier Science Publishers.

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