OFFPRINTS FROM BACTERIAL OUTER MEMBRANES AS MODEL SYSTEMS
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CHAPTER 8

Model Membrane Studies of Porin Function

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1. INTRODUCTION

The outer membrane of gram-negative bacteria has many unique properties that distinguish it from other biological membranes (see reviews by DiRienzo *et al.*, 1978; Inouye, 1979; Nikaido and Nakae, 1979). In particular, it serves a barrier function, excluding hydrophobic and large hydrophilic substances but allowing the passage of hydrophilic compounds smaller than a given size (the exclusion limit). As a result of the research of Nakae and Nikaido, we now know that this molecular sieving property of outer membranes is due to a class of proteins that the researchers termed *porins* (Nikaido and Nakae, 1979). These porin proteins form channels across the outer membrane, allowing substrate diffusion into the periplasm. In this review, a broad definition of porins will be used to include any outer membrane protein capable of mediating relatively nonspecific diffusion, even if under some circumstances there is evidence that the physiological function of the protein involves solute selectivity.

Porins have been the subject of many studies that have examined their regulation, biosynthesis, structure, activity as phage receptors, and immunochemistry. None of these aspects is considered here, since they are either discussed in depth in other chapters of this book (e.g., Chapters 1, 2, 3, 4, 5, 6, 7) or have been reviewed in depth elsewhere (DiRienzo *et al.*, 1978; Inouye, 1979; Nikaido, 1979, 1983; Nikaido and Nakae, 1979; Osborn and Wu, 1980; Lugtenberg, 1981; Lugtenberg and Van Alphen, 1983; Hancock, 1984). In addition, no attempt will be made to apply a historical perspective to the research on porins, since this too has been discussed previously (Nikaido and Nakae, 1979; Nakae, 1986) and since functional investigations of porins started only in 1975. Instead, this chapter discusses the development of model membrane systems for studying porin function ex vivo, the types of information obtainable (and that obtained) from such model membrane studies, and the relevance of these porin studies to other transport systems.

2. PURIFICATION AND PHYSICAL PROPERTIES OF PORIN PROTEINS

2.1 Purification Procedures

Two properties common to many porins have allowed fairly simple purification procedures to be applied to the proteins. The first of these is that most porins remain insoluble in the strongly denaturing ionic detergent SDS at low temperatures, presumably due to their strong noncovalent association with the

peptidoglycan layer (Rosenbusch, 1974; Lugtenberg and Van Alphen, 1983). This allows removal, by solubilization in SDS, of a substantial number of potential contaminants. The porin can then be eluted from the peptidoglycan and solubilized by a combination of high salt (0.1–0.5 M NaCl), 10 mM EDTA (to prevent LPS-mediated aggregation?), and 2% SDS (Rosenbusch, 1974; Nakae, 1979; Nikaido, 1983). The second important property is the stability of porin tertiary and quaternary structure to SDS treatment at moderate temperatures. Thus, although porins have molecular weights similar to those of the major contaminants observed after the aforementioned differential SDS solubilization procedure, the maintenance of their trimer structure in SDS, and consequent larger molecular size, allows simple separation from all contaminating peptides by gel sieving chromatography on Sepharose 4B (Nikaido, 1983). Of course, when more than one porin species is present in a single cell it is extremely difficult to separate and purify them by this or other procedures. However, the availability, in a number of bacteria, of mutants with single porin species has allowed this technique to be used for 19 of the porin proteins described in Table 1.

In some cases, alternative procedures have been applied. For *Pseudomonas aeruginosa* porin proteins F and D1, solubilization of outer membrane proteins in Triton X-100 with (protein F) or without (protein D1) lysozyme digestion of the peptidoglycan, followed by ion exchange chromatography has yielded quite pure preparations of porin (Hancock *et al.*, 1979; Hancock and Carey, 1980). Other procedures have involved differential solubilization of outer membranes using a variety of different detergents and subsequent chromatographic separations of the detergent extracts (Young *et al.*, 1983; Bavoil *et al.*, 1984; Douglas *et al.*, 1984; Gabay *et al.*, 1985).

2.2 Physical Properties of Porins

A variety of physical properties that have been studied for a range of porin proteins are summarized in Table 1. In general, porins have monomer molecular weights in the range 28,000–48,000, are present in the membrane as native trimers that are resistant to SDS denaturation, demonstrate a high content of β-sheet structure (which is also resistant to SDS denaturation), are strongly but noncovalently associated with the underlying peptidoglycan and with LPS (see Table 1 legend for references), and have acidic pIs (Lugtenberg and Van Alphen, 1983). While exceptions to these general rules have been noted, porins seem to form a fairly tight and unique class of membrane proteins, perhaps suggesting that they have a common evolutionary origin. Certainly in the case of the *Escherichia coli* OmpC, OmpF, and PhoE porins, a substantial homology in nucleotide and amino acid sequences has been observed (Mizuno *et al.*, 1983; Overbeeke *et al.*, 1983). In addition, OmpF and OmpC porins show immunological cross-reactions with proteins of similar molecular weight from other *Enterobacteriaceae* (Hofstra and Dankert, 1979; Overbeeke and Lugtenberg, 1980). In contrast, very little sequence homology

TABLE 1. Physical Properties of Porin Proteins

Porin Protein	Species	Molecular Weight (monomer)	Native Oligomeric State	β-Sheet Structure	SDS Stable Oligomers	Peptidoglycan Associated	LPS Associated	Channel Diameter (nm)	Conditions Favoring Production	Genetic Map Position	Other Names
OmpF	*E. coli*	32,705	Trimer	+	+	+	+	1.15	Low osmolarity	20.7	Ia, b, 09, 1a
OmpC	*E. coli*	36,000	Trimer	+	+	+	+	1.02	High osmolarity	47.1	Ib, c, 08, 1b
LamB	*E. coli*	47,932	Trimer	+	+	+	+	1.4	Maltose	91.0	
PhoE	*E. coli*	36,782	Trimer		+	+	+	1.1	Pi-limitation	5.9	E, Ic
NmpC	*E. coli*	39,500			+	+		1.0		12	
K	*E. coli*	40,000			+	+		1.1	Capsule production		
Lc	*E. coli*	36,500			+	+			Phage PA2 lysogeny	Phage	2
OmpF	*S. typhimurium*	39,300	Trimer	+	+	+	+	1.2	Low osmolarity	21	35K, 39K
OmpC	*S. typhimurium*	39,800	Trimer	+	+	+	+	1.3	High osmolarity	46	36K, 40K
OmpD	*S. typhimurium*	38,000	Trimer	+	+	+	+	1.3		28	34K, 38K
PhoE	*S. typhimurium*	34,000				+			Pi-limitation		
37kD	*K. pneumoniae*	37,000				+		1.2			
39–40kD	*K. pneumoniae*	39,000				+		1.6			
E	*Y. pestis*	33,000			+	+		1.0	Low growth temperature		
F	*P. aeruginosa*	39,000	Trimer	+	−(+)	+	+	2.0			
P	*P. aeruginosa*	48,000	Trimer		+	−	+	0.6	Pi-limitation		
D1	*P. aeruginosa*	46,000	Trimer		−	−	+		Glucose		
I	*N. gonorrhoeae*	34,000	Trimer		−			1.0			

I	*N. meningitidis*	≃37,000			+/−		−	2.1
42kD	*A. salmonicida*	42,000			+	+		1.0
43kD	*A. hydrophila*	43,000			+	+		1.0
47kD	*R. sphaeroides*	47,000		+	+	+		1.2
43kD	*R. capsulata*	43,000		+	+	+		1.6
40kD	*H. influenza*	40,000			−			~1.4
MOMP	*L. pneumophilia*	28,000			+	+	−	1.0
MOMP	*C. trachomatis*	39,500			+	+		
Group 2	*Brucella* sp.	37,000–42,000	Trimer (?)		+	+	+	1.2
33kD	*P. denitrificans*	33,000			+	+		≃1.7
32kD	*S. aurantia*	32,000	Trimer (?)		+	+	−	2.3

Source: References to the data, listed by porin, are OmpF, OmpC (*E. coli*) (Mizuno *et al.*, 1983; Nakae *et al.*, 1979; Rosenbusch, 1974; Kameyama *et al.*, 1982); LamB (Schwartz, 1983; Neuhaus, 1982; Nikaido and Nakae, 1979); PhoE (Angus and Hancock, 1983; Lugtenberg and Van Alphen, 1983; Benz *et al.*, 1984); NmpC (Hindahl *et al.*, 1984; Pugsley and Schnaitman 1978a; Lee *et al.*, 1979); K (Sutcliffe *et al.*, 1983; Whitfield *et al.*, 1983); Lc (Pugsley and Schnaitman, 1978b; Lee *et al.*, 1979); OmpF, OmpC, OmpD (*S. typhimurium*) (Tokunaga *et al.*, 1979b,c; Nakae and Ishii, 1978; Kuusi *et al.*, 1981; Ishii and Nakae, 1980); PhoE (*S. typhimurium*) (Brass *et al.*, 1985); 37kD (Lugtenberg *et al.*, 1977; Sawai *et al.*, 1982; Kaneko *et al.*, 1984); 39–40 kD (Lugtenberg *et al.*, 1977; Sawai *et al.*, 1982; Kaneko *et al.*, 1984); E (Darveau *et al.*, 1983); F (Hancock and Carey, 1979; Hancock *et al.*, 1978; Mizuno and Kageyama, 1979); P (Hancock *et al.*, 1982; Angus and Hancock, 1983); D1 (Hancock and Carey, 1979, 1980; Angus and Hancock, 1983); I (*N. gonorrhoeae*) (Douglas *et al.*, 1981; Leith and Morse, 1980; McDade and Johnston, 1980; Blake and Gotschlich, 1982); I (*N. meningitidis*) (Poolman *et al.*, 1980); 42kD (Darveau *et al.*, 1984); 43kD (*A. hydrophila*) (Darveau *et al.*, 1984); 47kD (Weckesser et al., 1984); 43kD (*R. capsulata*) (Flammann and Weckesser, 1984); 40kD (Vachon *et al.*, 1985); MOMP (*L. pneumophilia*) (Gabay *et al.*, 1985; Hindahl and Iglewski, 1984); MOMP (*C. trachomatis*) (Bavoil *et al.* 1984); group 2 (Douglas *et al.*, 1984; Verstreate *et al.*, 1982); 33kD (Zalman and Nikaido, 1985); and 32kD (A.M. Kropinski, T.R.Parr, W.C. Ghiorse, B. Angus, R.E.W.Hancock and E.P. Greenberg, manuscript submitted).
Note: The full species names of the bacteria listed are *Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae* (listed in the original papers as *Enterobacter cloaceae* but recently reidentified; T. Sawai, personal communication), *Yersinia pestis, Pseudomonas aeruginosa, Neisseria gonorrhoeae, Neisseria meningitidis, Aeromonas salmonicida, Aeromonas hydrophila, Rhodopseudominas sphaeroides, Rhodopseudomonas capsulata, Haemophilus influenza, Legionella pneumophilia, Chlamydia trachomatis, Brucella* sp., *Paracoccus denitrificans*, and *Spirochaeta aurantia*.

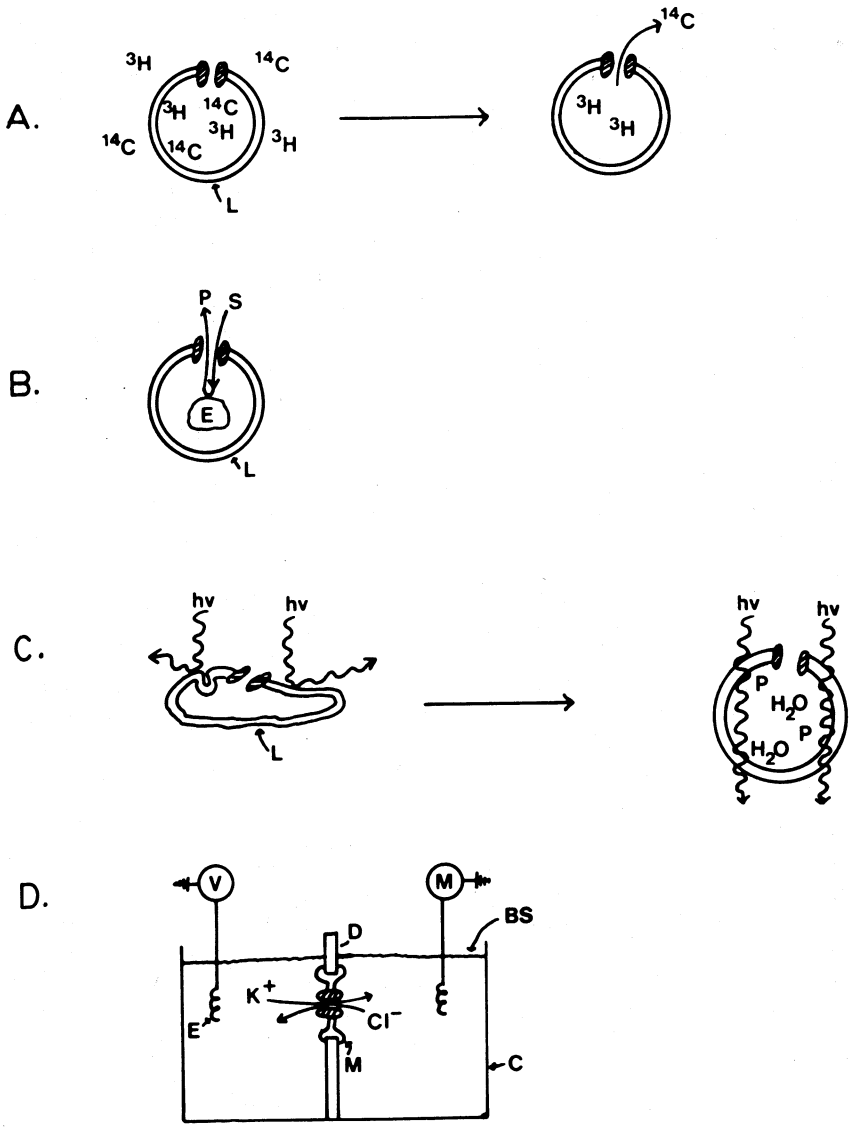

Figure 1. Schematic representation of the four model membrane systems described in Section 3. (*a*) Proteoliposome radioisotope efflux (Section 3.1). Liposomes (L) are reconstituted from phospholipids, LPS, and porin proteins (shaded ovals) in the presence of a ^{3}H dextran of high molecular weight (20,000–70,000) and ^{14}C sucrose (342 daltons). Dilution of the medium external to the liposomes creates a concentration gradient across the liposome membrane, allowing ^{14}C sucrose to leak out. ^{3}H-dextran, being larger than the exclusion limit of the porin, is retained within the liposome. (*b*) Enzyme-containing proteoliposome assay (Section 2.2). Liposomes (L) are reconstituted in the presence of an enzyme (E) and freed from external enzyme by gel sieving chromatography. Permeability is measured by assessing the rate of conversion of substrate (S) to product (P). Note that although the liposomes shown here are depicted as unilamellar, it is

between the LamB and OmpF porins has been observed, despite the substantial similarities in properties (Table 1). It will be of great interest in the future to determine if porin genes have evolved through divergent or convergent evolutionary processes, since it seems that this group of proteins offers an ideal opportunity for studying evolution at the molecular level.

3. METHODOLOGIES FOR MODEL MEMBRANE STUDIES OF PORINS

A variety of reconstitution systems have been used to demonstrate the pore-forming function of porins in vitro. These are summarized in conceptual form in Figure 1. The techniques are described below in outline, with emphasis on the types of information that can be obtained and the problems that can be encountered in obtaining and analyzing data from these systems. For details of the methods, specific references are given in each section for the appropriate method.

3.1 Radioisotope Efflux Using Proteoliposomes

The use of proteoliposomes reconstituted from porin protein, LPS, and phospholipids was pioneered by Nakae (1975) and has been described in detail by Nikaido (1983). Briefly, phospholipids, LPS, and porin are dried onto the surface of a glass tube. They are then resuspended in a solution containing a small ^{14}C-labeled sugar (the permeant solute) and a large ^{3}H-labeled polysaccharide (the impermeant control solute), such as a dextran. Liposomes en-

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almost certain that they (as well as the liposomes depicted in *c*) are multilamellar. (*c*) Liposome swelling assay (see Section 2.3). Proteoliposomes are reconstituted in the presence of an impermeant solute of high osmolarity. These proteoliposomes (which are actually multilamellar) are not of uniform shape and are thus capable of efficiently scattering light (hv) (i.e., they will have a measurably high adsorbance). Upon addition to the external milieu of a permeant solute (P) of equivalent osmolarity to that of the internalized impermeant solute, a concentration gradient of permeant solute across the liposome membrane is created. The permeant solute thus flows through the porin into the liposome, resulting in increased osmolarity. Consequently, water (H_2O) flows in to maintain the iso-osmolarity of the liposome interior and exterior. This results in liposome swelling (i.e., expanding to a spherical shape), at least for the most external liposome bilayer, and thus the liposome is less able to scatter light. The rate of swelling is related to the rate of solute influx. (*d*) Black lipid bilayer assay (see Section 2.4). The apparatus involves a Teflon chamber (C) divided into two compartments by a Teflon divider (D) containing a small hole. The chamber is filled with a bathing salt solution (BS), and electrodes (E) dip into the salt solutions in the two compartments. One electrode is connected to a voltage source (V), whereas the other is connected to a measuring device (M, actually a current amplifier to boost the measured currents 10^9-fold and a chart recorder). The circuit is completed through earth. A lipid membrane (M) is formed across the hole in the Teflon divider, and reconstitution of single porin molecules into the membrane can be followed by current increases due to movement of ions (K^+ and Cl^- in this case) through the porin channels.

capsulating these radioactive saccharides are reconstituted by scraping the protein-LPS-phospholipid film from the sides of the glass tube, dispersing these components by vortexing and sonication, and heating the suspension to 45°C, followed by cooling. Dilution of the solution containing the liposomes creates a concentration gradient across the liposome membranes, and the permeant ^{14}C sugar preferentially diffuses out of the liposome, according to Fick's law (equation 1, Section 3.5). The liposomes are then collected by filtration or column chromatography, and the relative retention of ^{14}C-permeant sugar and ^{3}H-impermeant dextran is assessed.

The assay is relatively uncomplicated, although the level of detergent contaminating the porin preparation (Hancock *et al.*, 1979) and concentration of magnesium at the time of reconstitution (Vachon *et al.*, 1985) may be critical. With some solutes, almost complete efflux or retention is observed; with others, however, an intermediate degree of retention may be observed (Fig. 2). The latter observation may be related to either the heterodispersity of the solute (as seen for dextrans larger than 1,500 daltons), variations in the size of individual pores (see Benz *et al.*, 1978), or the incompleteness of the efflux process, in the given time, for solutes approaching the pore exclusion limit. In addition, the retention of impermeant saccharides relative to the amount added to the liposome reconstitution assay increases with increasing molecular size (see, e.g., Hancock *et al.*, 1979), for as yet undertermined reasons.

These complications aside, this assay is capable of providing two types of information: it can provide definitive proof that a given purified protein is a porin, and it can be used to determine the approximate exclusion limit of the

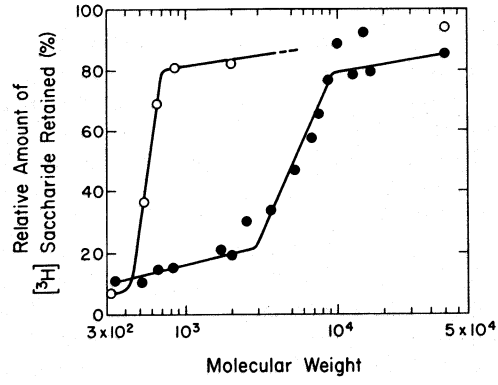

Figure 2. Typical data obtained using the radioisotope efflux assay to measure the exclusion limits of *P. aeruginosa* outer membranes (filled circles; similar data were obtained using purified protein F) and *S. typhimurium* porin (open circles). The assay was performed as described in Section 3.1 and Figure 1a. The results imply that *P. aeruginosa* porin has a larger exclusion limit than does *S. typhimurium* porin, since after dilution certain saccharides (molecular weights 8,000–2,000 daltons) retained in proteoliposomes containing the latter porin were able to leak into the medium of proteoliposomes containing *P. aeruginosa* porin. (*Source*: Reprinted with permission from Hancock and Nikaido, 1978.)

porin (Fig. 2) for comparative purposes. A summary of data obtained by this method for different porins is included in Table 2.

3.2 Substrate Uptake into Enzyme-Containing Proteoliposomes

Nakae and colleagues (Tokunaga *et al.*, 1979b) have reconstituted liposomes from phosphatidyl choline and porin protein (in the absence of added LPS). An enzyme (either α-glucosidase, trypsin, or alkaline phosphatase) is included in the solution in which the liposomes are reconstituted by sonication of resuspended phospholipid-porin films. Thus, the liposomes should enclose a portion (0.3%) of the enzyme. Liposomes are freed from unincorporated enzyme by gel sieving chromatography. The rate of uptake of various substrates into proteoliposomes is determined as the rate of hydrolysis and a permeability coefficient (called PA and equal to $DA/\Delta x$ in equation 1, Section 3.5) calculated according to Fick's law.

The method is conceptually simple but not without problems. First, the liposomes made by this technique are probably multilamellar. If so, the calculations of the substrate concentration inside the liposomes at steady state will be inaccurate, since this is performed by application of the Michaelis-Menten equation ($V = C_i V_{max}/K_m + C_i$, where V = the rate of hydrolysis by enzyme inside the liposomes, C_i is the substrate concentration inside the liposomes, and V_{max} and K_m are the Michaelis-Menten constants obtained from kinetic experiments with Triton X-100–dispersed liposomes). Thus, if not all of the enzyme is available to the substrate, as expected in multilamellar liposomes, the estimate of V_{max} and thus of C_i (used in the calculation of the permeability coefficient) will be incorrect. In addition, as pointed out by Tokunaga and coworkers (1979b), the permeability of various substrates may be compared with each other only when the K_m is the same. Also, the presence of even a tiny fraction ($< 0.1\%$) of the enzyme bound to the surface of the liposome can significantly influence the calculated permeability coefficients. Finally, the method assumes identical porin concentrations per unit area of lipid membrane and identical liposome sizes per unit encapsulated enzyme for lateral comparisons (between liposomes reconstituted from different species of porins or chemically modified species of porins) to be made. Although the method suffers from some potential technical difficulties, information obtained by this technique for chemically modified porins (Tokunaga *et al.*, 1981) and for maltose oligomer diffusion through LamB porin (Ishii *et al.*, 1980) has been confirmed in principle by other reconstitution techniques (Luckey and Nikaido, 1980; Benz *et al.*, 1984).

3.3 Liposome Swelling

Bangham and colleagues (1967) introduced a technique involving optical measurements of the osmotic swelling of liposomes as a method of estimating the rate of uptake of various amphiphilic substances. Nikaido and Rosenberg

TABLE 2. Properties of Porin Channels Obtained from Experiments Involving Extensive Washing of Liposomes with Porins Incorporated into the Liposome Bilayers

Porin	Bacterial Species	Exclusion Limit for Saccharides (daltons)	Amount of Protein Required for Reconstitution[a] (μg/μmol lipid)	References
OmpF	*E. coli*	~600	10	Nakae, 1976
LamB	*E. coli*	~600	19–100	Nakae, 1979; Luckey and Nikaido, 1980
OmpF	*S. typhimurium*	~600	10	Nakae and Ishii, 1978
OmpC	*S. typhimurium*	~600	10	Nakae and Ishii, 1978
OmpD	*S. typhimurium*	~600	10	Nakae and Ishii, 1978
F	*P. aeruginosa*	~6,000	60	Hancock *et al.*, 1979
Outer membranes	*P. aeruginosa*	~6,000	260	Hancock and Nikaido, 1978
Dl	*P. aeruginosa*	>600, <5,000	100	Hancock and Carey, 1980
40kD	*H. influenzae*	1,400	20	Vachon *et al.*, 1985
Group 2	*Brucella sp.*	~600–800	10	Douglas *et al.*, 1984

[a]Required to give leakage of five-sixths or more of the ^{14}C sucrose out of the proteoliposomes upon dilution.

(1983; Nikaido, 1983) modified this method extensively for the assay of uptake through porins. In principle, liposomes are reconstituted from phosphatidyl choline containing 3.2 mole % dicetylphosphate by resuspension of the lipid from a dried film into an aqueous solution of the purified protein, followed by sonication. The liposomes are then dried down again as a film on a glass tube and resuspended in a solution containing stachyose, NAD, and imidazole-NAD buffer pH 6.0, by hand shaking. Small aliquots (10–20 μl) are then diluted into 0.6 ml of the test solutions (which are carefully adjusted to be isotonic with the internal contents of the liposomes, i.e., the impermeant sugar stachyose). If the solute in the test solution is incapable of permeating through the porin pores, the system will be at osmotic equilibrium. However, since the liposomes do not contain the solute, a chemical potential gradient across the membrane will drive the uptake of permeant solutes. As the solute is taken up, it will be followed by influx of water to maintain the osmotic equilibrium (by "diluting" the internal contents of the liposomes). As the liposomes swell, their refractive indexes and hence their abilities to scatter light are reduced. Thus, the measured optical density of the suspension will decrease (Fig. 3). It is assumed that the initial rate of optical density decrease will reflect the solute penetration rate into the outermost layer.

The technique has been widely used for the study of the properties of porins, particularly of *E. coli* porins (Nikaido and Rosenberg, 1983; Yoshimura *et al.*, 1983). Nevertheless, the technique has a variety of possible complications. First, Bangham and associates (1967) have discussed that the change in optical density may be influenced by light scattering or refractivity of the solutes, and this must be carefully controlled for in liposome swelling experiments (see Nikaido, 1983, for discussion). It is less easy to regulate the possible complication of hydrodynamic interactions between the liposome and the solute.

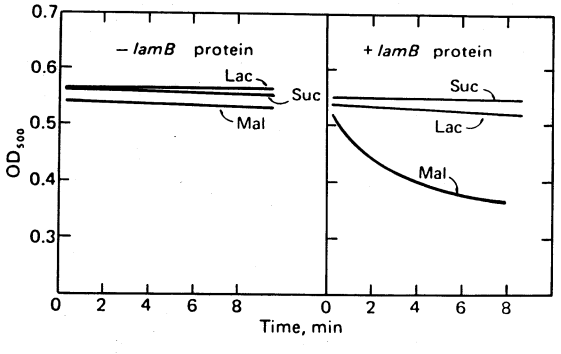

Figure 3. Proteoliposome swelling in the presence of different disaccharides of molecular weight 342. Proteoliposomes were reconstituted with or without LamB porin as described in Section 3.3 and Figure1*c*. Note that the LamB-containing liposomes swell more rapidly in maltose than in sucrose or lactose, suggesting a preference of this channel for maltose. (*Source*: Reprinted with permission from Luckey and Nikaido, 1980.)

Second, Bangham and coworkers (1967) performed their measurements of changes in optical density using a stop-flow apparatus and showed that even at slow swelling rates, the curves of optical density decrease over time departed from linearity after less than 5–10 s (see, e.g., Fig. 3 for typical tracings). This considerably complicates the measurements, although Nakae (1985) has proposed a mathematical manipulation that corrects in part for this. Third, the substantial variability (four- to eight-fold) in the rates of swelling due to disaccharides of identical molecular weight (Nikaido and Rosenberg, 1983) complicates the estimation of channel size by fitting the data to the Renkin equation (as illustrated by Fig. 5 of Nikaido and Rosenberg, 1981). Fourth, a variety of potential complications, including a nonlinear dependence of swelling rate on porin concentration seen for OmpC (Nikaido and Rosenberg, 1983), charge on the impermeant sugar contained within the liposomes (Nikaido and Rosenberg, 1983), and others discussed in detail by Nikaido (1983), suggest that great care must be taken in the performance and interpretation of these assays.

Despite these problems, the liposome assay allows an estimate of relative diffusion rates of a variety of solutes and consequently can be used to determine a variety of types of information in addition to providing a simple confirmation of the identity of a protein as a porin (Table 3). By studying the behavior of a variety of solutes of differing molecular weights an estimate of pore diameter can be obtained (see Section 3.5). Also, the relative swelling rates of liposomes in the presence of similar solutes differing in charge allows qualitative statements about the charge selectivity of channels. In addition, since swelling rates are related to porin concentration, an estimate of the relative efficiency (or "openness") of individual porins can be obtained (see Section 5.3). Data obtained for different porins using this procedure are summarized in Table 3.

3.4 Black Lipid Bilayer Studies

A procedure for the measurement of the conductivity of ions through porin pores is the black lipid bilayer technique (Benz and Hancock, 1981). The apparatus includes a Teflon chamber divided into two compartments by a Teflon wall that contains a small (0.1–2 mm^2) hole connecting the compartments. Electrodes dip into the aqueous solutions on either side of the hole. A membrane is formed across the hole by the painting of a solution of lipid in a solvent such as n-decane across the hole. The lipid thins out in the center of the hole until it forms a bilayer that is optically black when viewed in incident light. The membrane is almost impermeable to ions, and, thus, application of a voltage across the naked membrane bilayer does not result in a substantial current. If porin is added in detergent solution to one side of the membrane or the other, stepwise conductance fluctuations are observed (Fig. 4; note that conductance is current per unit of applied voltage). By analogy with previous studies of pore-forming compounds, these conductance fluctuations can be interpreted as the stepwise incorporation of single pore-forming oligomers into the membrane. The observed increase in current is small (around 10^{-11}–10^{-12}

TABLE 3. Properties of Porin Channels Derived from Proteolysosome Swelling Assays

Porin	Bacterial Species	Classes of Permeant Substances Demonstrated	Inferred Ion Selectivity of the Channel	Estimated Channel Diameter (nm)	Amount of Porin Required to Give a Standard Swelling Rate[a] (μg)	References
OmpF	*E. coli*	Antibiotics, saccharides, peptides	Cation	1.16	0.02	Nikaido and Rosenberg, 1983
OmpC	*E. coli*	Antibiotics, saccharides, peptides	Cation	1.08	—	Nikaido and Rosenberg, 1983
PhoE	*E. coli*	Antibiotics, saccharides, peptides	Anion	1.06	—	Nikaido and Rosenberg, 1983
LamB	*E. coli*	Saccharides, amino acids	—	—	—	Luckey and Nikaido, 1980
37kD	*K. pneumoniae*[b]	Saccharides, antibiotics	Cation	1.2	—	Kaneko *et al.*, 1984
39–40kD	*K. pneumoniae*[b]	Saccharides, antibiotics	—	1.6	—	Kaneko *et al.*, 1984
F	*P. aeruginosa*	Saccharides	—	2.0	0.6	Yoshimura *et al.*, 1983
I	*N. gonorrhoeae*	Saccharides	—	—	—	Douglas *et al.*, 1981
47kD	*R. sphaeroides*	Saccharides	—	1.24	≃0.02–0.1[c]	Weckesser *et al.*, 1984
43kD	*R. capsulata*	Saccharides	—	1.60	≃0.02[d]	Flammann and Weckesser, 1984
MOMP	*C. trachomatis*	Saccharides	—	—	8	Bavoil *et al.*, 1984
Group 2	*Brucella abortus*	Saccharides	—	≃1.2	≃0.05	Douglas *et al.*, 1984
33kD	*P. denitrificans*	Saccharides	—	≃1.7	0.12[e]	Zalman and Nikaido, 1985

[a]Standard swelling rate is defined as an optical density change at 500 nm of 0.1 unit/min using proteoliposomes reconstituted with 2.25–2.40 μmol of phosphatidyl choline and 0.1 μmol of dicetylphosphate and using arabinose as the swelling agent.
[b]Originally described as *E. cloaceae* 206 but recently reidentified as *Klebsiella pneumoniae* (T. Sawai, personal communication).
[c]The lower estimate is extrapolated from data for oligomers in the indicated reference. The upper estimate is obtained for porin monomers.
[d]Similar to *E. coli*.
[e]Extrapolated from the observation that it took six times as much *P. denitrificans* cell envelope to give the same swelling rate as 5 μg of *E. coli* cell envelope (Zalman and Nikaido, 1985).

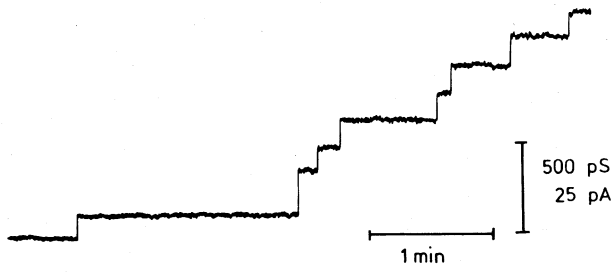

Figure 4. Stepwise conductance increases after addition of small amounts (10^{-11} M) of PhoE porin to the salt solution (0.1 M KH_2PO_4) bathing a lipid bilayer membrane. The individual steps represent single porin trimers reconstituting into the membrane and giving rise to conductance increases as described in Section 3.4 and Figure 1*d*. (*Source*: Reprinted with permission from Benz *et al.*, 1984.)

A) and must be substantially boosted by a current amplifier for recording on a chart recorder. A somewhat modified setup is used to measure the aggregate properties of 1,000 or so channels per membrane in macroscopic conductance experiments (Benz and Hancock, 1981). Schindler and Rosenbusch (1978) used a different method of porin incorporation and bilayer formation; this is discussed in detail in Section 4.5. Lynch and colleagues (1984) used basically the same procedure as that described above except that they used an osmotic gradient across the membrane to force the fusion of porin-containing vesicles with the black lipid bilayer.

This method has the disadvantage that only the permeability of charged ions can be studied. In addition, since incorporation of protein into the black lipid bilayer membrane involves less than 1 in 10,000 molecules introduced into the chamber, no quantitative statements about the relative degree of activity of individual porins can be made. The requirement for specialized apparatus somewhat restricts the general usage of this technique.

Nevertheless, this technique affords single-molecule sensitivity and is the most powerful technique discussed in this section for describing the molecular anatomy of the interior of porin channels. It is capable of providing an estimate of the channel diameter (see Section 3.5), a precise measurement of the ion selectivity for a variety of anions and cations (see Section 4.3), a measurement of the heterogeneity of individual channels (see Section 4.2), information about ion binding sites in the channel (see Section 5.2), and a determination of whether the channel is voltage regulated (see Section 4.5). Data obtained from black lipid bilayer studies of different porins is summarized in Table 4.

3.5 Some Important Equations

Since the analysis of porin channel properties in model membrane systems involves mathematical manipulation of data based on a variety of assumptions, these will be briefly discussed below. For a fuller discussion of the theories

TABLE 4. Single-channel Conductance in 1 M NaCl and Selectivity of Bacterial Porin Proteins

Bacterium	Pore	Selectivity at pH 7 (Permeability for K^+/ Permeability for Cl^-)	Single-Channel Conductance in 1 M KCl (nS)	Estimated Pore Diameter (nm)[a]
P. aeruginosa	P	<0.01	0.25	0.6
	F	2.7	5.6	2.0
E. coli	OmpF	3.7	2.1	1.2
	OmpC	13.4	1.5	1.0
	PhoE	0.33	1.8	1.1
	LamB	—	0.16	—
	NmpC	0.27	1.3	1.0
	K	15.1	1.8	1.1
S. typhimurium	OmpD (38K)	22.6	2.4	1.3
	OmpF (39K)	13.6	2.2	1.2
	OmpC (40K)	41.0	2.4	1.3
	PhoE	0.54	—	—
Y. pestis	E	12.6	1.4	1.0
A. salmonicida	42kD	7.5[b]	1.6	1.0
A. hydrophila	43kD	—	1.3[c]	1.0
N. gonorrhoeae	I	0.3	1.4[c]	1.0
N. meningitidis	I	0.8[c]	6.0[c]	2.1
L. pneumophilia	MOMP	4.0	~1.0[c]	1.0
S. aurantia[d]	32kD	—	7.7	2.3
A. variablis	40–80kD	1.7	3.5	1.6

Source: Data from Benz and Böhme, 1985; Benz and Hancock, 1981; Benz *et al.*, 1980a,b, 1982, 1983, 1984a,b, 1985; Brass *et al.*, 1985; Young *et al.*, 1983; Lynch *et al.*, 1984; Darveau *et al.*, 1983, 1984; Hindahl *et al.*, 1984; and Gabay *et al.*, 1985.

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

[b]R.E.W. Hancock and R. Benz, unpublished data.

[c]Data for 1 M NaCl, which usually give lower single-channel conductances and higher anion selectivity than do data for KCl, due to the higher hydration and consequent lower mobility of Na^+ ions. The single-channel conductance for *L. pneumophilia* was extrapolated from data for conductivity in 100 mM NaCl.

[d]A.M. Kropinski, T.R. Parr, W.C. Ghiorse, B. Argus, R.E.W. Hancock, and E.P. Greenberg, manuscript submitted.

described below, the reader is referred to the following reviews (Renkin, 1954; Lakshminarayanaiah, 1969; Bockris and Reddy, 1970; Macey, 1980).

Fick's First Law of Diffusion. The descriptive basis of diffusion can be traced back to the theoretical work of Adolf Fick, published in 1855. He showed that the diffusion rate (dm/dt, where m is the mass of material being transferred and t is the time) is proportional to the area, A, of the plane through which the transfer is being made and the concentration gradient ($\Delta C/\Delta x$, where ΔC is the difference in concentrations of the material over a distance Δx). Thus,

Fick's first law of diffusion states

$$\frac{dm}{dt} = \frac{DA}{\Delta x}\Delta C \tag{1}$$

where D is the proportionality coefficient known as the *diffusion coefficient.* (Note that D is not a constant, since it increases slightly toward a limiting value as the solution is diluted. At infinite dilution, $D = RT/N6\pi\eta r$, where R is the gas constant, T is the absolute temperature, N is Avogardro's number, π has its usual mathematical meaning, η is the solution viscosity, and r is the hydrated radius of the diffusing solute.) For the channels of porin molecules inserted into a lipid bilayer membrane, A becomes the total area of porin channels available for diffusion, Δx the length of the porin channel (approximately 6 nm for the OmpF porin; Dorset *et al.,* 1984), and ΔC the concentration gradient across the membrane.

Calculation of Pore Size. If one knows the number of functional channels and assumes that the pores are uniform cyclinders oriented perpendicularly to the surface and that the pore interiors interact minimally with the molecule whose diffusion is being measured, then one can use Fick's law to calculate pore area. In fact, neither of these assumptions is correct, although the number of porin molecules per unit area of membrane can be calculated. Thus, Nikaido and Rosenberg (1981, 1983) applied the considerations adopted by Renkin (1954), who corrected for steric hindrance (the Ferry correction, which establishes the condition that for entrance to the pore a molecule must pass through the opening without striking the edge) and for friction between a molecule moving within a pore and the walls of the pore (the Faxen correction). Thus, the total restriction to diffusion due to these combined effects is given by the equation

$$\frac{A}{A_0} = \left(1 - \frac{a}{r}\right)^2\left[1 - 2.104\left(\frac{a}{r}\right) + 2.09\left(\frac{a}{r}\right)^3 - 0.95\left(\frac{a}{r}\right)^5\right] \tag{2}$$

where A is the effective area of the opening, A_0 is the total cross sectional area of the channel, r is the radius of the pore, and a is the radius of the molecule in its solvated state. Renkin (1954) was able to demonstrate that application of this correction to diffusion data obtained for different compounds using porous cellulose membranes as model systems allowed reasonable estimates of channel radius. Consequently, Nikaido and Rosenberg (1981, 1983) applied equation 2 to the analysis of liposome swelling data to calculate pore radii. It should be pointed out that the calculation makes the assumption that porin channels are uniform cylinders of constant pore radius r (which is not correct; see Dorset *et al.,* 1984) and also relies on estimations of the

molecular radii of hydrated sugars that are somewhat disputed (see Renkin, 1954; Durbin, 1960).

An alternative method used to calculate pore size has been employed in electrical measurements of the average conductance, for a variety of salts, across a single channel of the given porin. This also makes the assumption that the pore is a perfect cylinder and that the ions move through the channels in the same way that they move through the aqueous solution on either side of the membrane in which the porin is inserted (the specific conductivity, σ, of a given solution is defined as the conductance, or current per unit voltage, of the solution between two 1-cm^2 areas placed 1 cm apart). Making this assumption,

$$\overline{\Lambda} = \sigma\pi r^2/l \tag{3}$$

where $\overline{\Lambda}$ is the average single-channel conductance, π has its usual mathematical meaning, r is the channel radius, and l is the length of the pore. Note that in addition to the incorrect assumption that channels are uniform cylinders, this model also assumes that the ions are unaffected by the considerations of frictional interactions with the walls of the channels or charged amino acid side chains within the channel. While these considerations are important and would suggest that radii obtained by application of equation 3 are underestimates, certain data suggest that this equation can be used with reasonable confidence. First, the same relationships that hold true for the specific conductance, σ (i.e., linear dependence on salt concentration, dependence on the nature of the anion and cation, and independence of applied voltage over a wide range of voltages), also hold true for the average single channel conductance (although as a caveat to this, it should be noted that for very large ions, such as $Tris^+Hepes^-$, there is often some restriction of mobility through the channel, as revealed by a lower $\overline{\Lambda}$ to σ ratio; see Benz *et al.*, 1978, 1980b). Second, many but not all porins show quite weak ion selectivity, suggesting that charged amino acid side chains only weakly influence the relative amounts of small ions within the channel at any given time (Benz *et al.*, 1980b, 1985).

A third method of estimating channel size is on the basis of the exclusion limit in liposome dilution experiments. This method merely suggests that the effective size of a channel is equivalent to a size slightly larger than the largest molecule capable of diffusing along a concentration gradient through the channel after infinite dilution of the liposomes (see Section 3.1). The problem with this method is that if molecules have ellipsoid rather than spherical geometry, then it may be argued that they could permeate through the channel, provided they were appropriately aligned relative to the channel, even though their apparent molecular sizes were larger than the pore size (as demonstrated empirically for the Hepes anion; Benz *et al.*, 1980b). In addition, uncertainties in the size or geometry of larger solute molecules makes the estimation of the

size of large channels, such as that of protein F of *P. aeruginosa,* extremely difficult by this method.

Despite the misgivings expressed above, channel size estimates do provide a descriptive basis for discussing and predicting pore properties. It is thus comforting that despite the substantial differences in the three techniques of pore size estimation and the different assumptions made in each, the estimates give quite similar values when applied to the same pores (Table 5).

Channel Selectivity. Two methods have been used to empirically demonstrate the general anion or cation selectivity of porin channels. These are discussed later. However one method, zero current potential measurements, allows calculation of the relative mobilities through these channels of specific anions and cations. A lipid bilayer apparatus is set up, as described in Section 3.4, and enough porin is added to the aqueous phase (a dilute salt solution, e.g., 0.1 M KCl) bathing the membrane to allow insertion of 10–100 porin molecules across the membrane (as determined by current measurements under an applied voltage). At this time, the applied voltage is removed and the measuring apparatus switched to allow measurement of potential (voltage) differences across the membrane. An aliquot of concentrated KCl is added to one side of the membrane (the concentrated side) and an equal aliquot of water to the other side (the dilute side). This creates a salt concentration gradient (i.e., a chemical potential across the membrane), which, as described by the Nernst equation, is a driving force for ion movement. Ions will thus tend to equilibrate across the membrane but, due to the ion selectivity of the channels, more of one ion species will move than of the ion with the opposite charge. If the channel is for example, cation selective, the preferential movement of cations will cause an increasing potential that is positive on the dilute side of the membrane. Ions will cease to move (zero current) when this electrical potential

TABLE 5. Comparison of Three Different Methods of Estimating Pore Diameters

Bacterium	Porin	Black Lipid Bilayer Studies (equation 3)	Liposome swelling Studies (equation 2)	Liposome Dilution Studies of the Exclusion Limit
E. coli	OmpF	1.15[a]	1.16[b]	1.13[d]
E. coli	OmpC	1.02[a]	1.08[b]	1.13[d]
E. coli	PhoE	1.11[a]	1.06[b]	1.13[d]
P. aeruginosa	Protein F	2.0[a]	2.0[c]	2.0–2.9[e]

[a]From the data of Benz *et al.*, 1985.
[b]Data of Nikaido and Rosenberg, 1983.
[c]Data of Yoshimura *et al.*, 1983.
[d]Based on estimates that raffinose (diameter 1.128 nm; Renkin, 1954) is the largest molecule capable of penetrating these *E. coli* pores (Nakae, 1976).
[e]Based on the estimate of the exclusion limit of protein F (3,000–9,000 daltons for heterodisperse dextrans; Hancock *et al.*, 1979) and the assumption that the spherical diameter of saccharides, including raffinose and these dextrans, is related to the cube root of their molecular weights (as discussed by Yoshimura *et al.*, 1983).

reaches a magnitude such that it exactly balances the opposing chemical potential. The measured electrical potential at this time is termed the *zero current potential.* Successive aliquots of concentrated salt will cause an increase in the zero current potential (Fig. 5). These increases in potential as a function of the salt concentration are related to the peremeabilities through the channel of the cation P_c and anion P_a and to the relative concentrations of these species on the concentrated side (C'') and the dilute side (C') of the membrane, as described by the Goldman-Hodgkin-Katz equation (see Lakshminarayanaiah, 1969, for the mathematical derivation of this equation), as follows:

$$V_m = \psi' - \psi'' = \frac{RT}{F} \ln \frac{P_c C'' + P_a C'}{P_c C' + P_a C''} \tag{4}$$

where V_m is the zero current potential; ψ' and ψ'' are the potentials on the dilute and concentrated side of the membrane, respectively; and R, T, and F are the gas constant, absolute temperature, and Faraday constant, respectively.

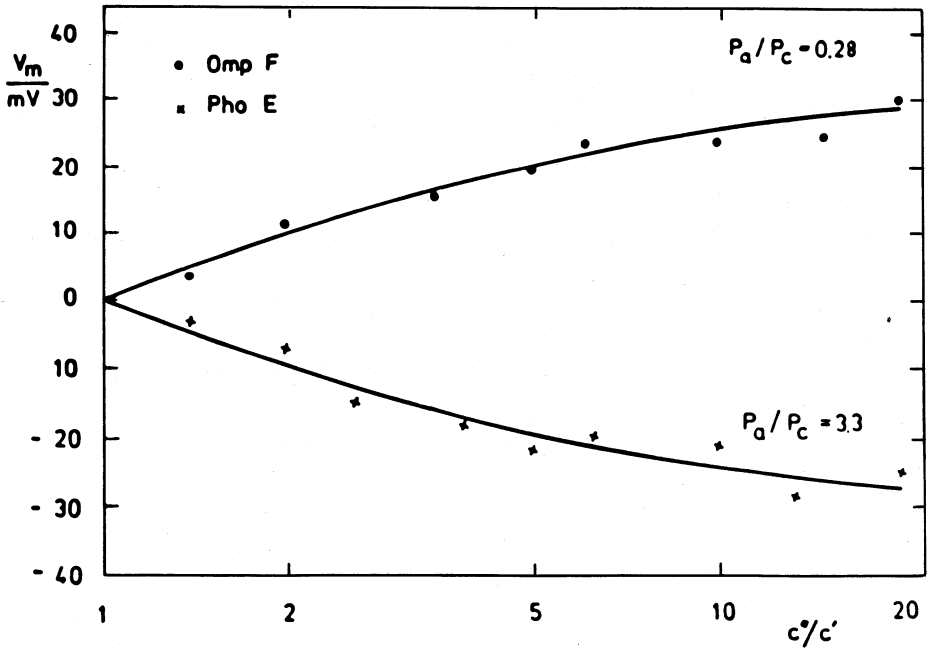

Figure 5. Demonstration of the selectivity for ions of the OmpF and PhoE porins. Zero current potentials (V_m), in response to increasing salt concentration gradients (c''/c') across lipid bilayer membranes containing about 100 porins, were recorded as described in Section 3.5. The lines were drawn according to the Goldman-Hodgkin-Katz equation, and the resultant relative permeabilities of the porins for anions (P_a) and cations were (P_c) determined. PhoE was found to be anion selective, whereas OmpF was cation selective. (*Source*: Reprinted with permission from Benz *et al.*, 1984.)

Note that for cation-selective channels V_m will become increasingly positive, whereas for anion-selective channels it will become increasingly negative (Fig. 5). The results of zero current potential measurements of ion selectivity for a variety of porins are summarized in Table 4.

4. PROPERTIES OF PORIN CHANNELS

The research described below is illustrative rather than comprehensive. However, many of the experiments described have been performed for a wide variety of porins. For more information regarding individual porins, the reader is referred to Tables 2–4 and to the source references in them.

4.1 Porin Channels Are Water-Filled

The first published experiments on porin reconstitution into liposomes (Nakae, 1975) clearly established that porin channels allowed the permeation of the hydrophilic disaccharide sucrose. The first independent confirmation of this data was obtained by Benz and colleagues (1978). They demonstrated that a variety of ions could permeate porin channels. The conductivity in 1 M KCl of a single OmpF porin channel was 112 mS/cm (assuming a 1.1-nm diameter cylindrical channel 6 nm in length; see Table 5). This is almost identical to the specific conductivity, σ, of a 1 M KCl solution in the absence of a membrane. In contrast, the conductivity in 1 M KCl of a lipid bilayer membrane that does not contain a porin protein is less than 1 μS/cm, or 5 orders of magnitude lower than OmpF porin channels. Similar data were obtained for 18 different salt solutions for which it was confirmed that the ratio of the single-channel conductance, $\bar{\Lambda}$, to the specific conductivity, σ, was relatively unaffected (i.e., < 3.5-fold) by the size, valence, or hydration of the ion despite a 30-fold variation in the specific conductivities (basically the ion mobilities or ability to carry a current) of these salt solutions (Table 6). In other words, the mobilities of these ions within the porin channels strongly reflected their mobilities in bulk solution in the absence of a membrane. This was further confirmed by demonstrating that (1) the single-channel conductance, $\bar{\Lambda}$, was directly proportional to the salt concentration of the aqueous phase bathing the membrane (Table 6), and (2) the current through a single channel was directly proportional to the applied voltage (Benz *et al.*, 1978).

Using the liposome swelling assay, Nikaido and colleagues demonstrated that large hydrophilic substances including sugars, peptides, and β-lactam antibiotics (Nikaido and Rosenberg, 1983; Yoshimura and Nikaido, 1985) permeated rapidly through porin pores. Furthermore, the rate of permeation of such molecules through porin channels was quite weakly influenced by charge (Nikaido and Rosenberg, 1983), a factor that dramatically influenced penetration into liposomes lacking porins (Bangham *et al.*, 1967). Also, the rate of diffusion of β-lactams into porin-containing liposomes was relatively in-

TABLE 6. Single-Channel Conductance Λ of *E. coli* B OmpF Porin Channels in Salt Solutions of Different Specific Conductivities (σ)

Salt	Concentration (M)	Λ (nS)	σ (mS/cm)	Λ/σ (10^{-8} cm)
KCl	3.0	4.3	250	1.72
	1.0	1.9	112	1.70
	0.3	0.54	34	1.58
	0.1	0.20	12	1.67
	0.03	0.078	4.6	1.69
NaCl	1.0	1.2	84	1.43
$MgSO_4$	0.5	0.24	33	0.73
$Tris^+Hepes^-$	0.5	0.064	7.2	0.89
$N(C_2H_5)_4^+Hepes^-$	0.5	0.032	4.8	0.67

Source: Benz *et al.*, 1978, 1979.

dependent of their hydrophobicity (with the possible exception of 5 or 6 of 14 monoanionic β-lactams, for which an inverse relationships was observed; Yoshimura and Nikaido, 1985), whereas the rate of penetration of such β-lactams into control liposomes lacking porin increased with increasing hydrophobicity (Yamaguchi *et al.*, 1982; Hiruma *et al.*, 1984). In addition, the influence of temperature on penetration rates through porin pores was consistent with a hydrophilic diffusion pathway (Nikaido and Rosenberg, 1981). All of these data strongly indicate that porin channels are water filled.

4.2 Porin Channels Are Large

As described in Section 3.5, it is possible to use a variety of procedures to directly measure the size of channels. Application of these procedures (Tables 3 and 4) suggests that porin channels range in size from 1.0 to 2.3 nm in diameter (except protein P; see Section 5.2). This is large enough to allow relatively free penetration of most growth substrates and requirements, including sugars, amino acids, small peptides, and inorganic ions. Reconstitution experiments have suggested that the OmpF porin channel (1.1 nm in diameter) will allow penetration of the trisaccharide raffinose (Nakae, 1976); the tetrapeptide tetraalanine (Nikaido and Rosenberg, 1983); and the large organic ions tetraethylamine, Tris, and Hepes (Table 6), but will exclude the tetrasaccharide stachyose (Nakae, 1976). It should be noted that although this and other reviews on porins have emphasized channel diameter as a definitive descriptive characteristic, there is evidence for all of the porins described in Table 4 that individual channels are heterogeneous, with single-channel conductances (that presumably reflect channel diameter) varying over a two-fold range. This may help to explain the observations of Nakae (1976) and others, who showed that, for example, raffinose was partially retained by OmpF-porin–containing proteoliposomes.

4.3 Porin Channels Are Usually Weakly Ion Selective

As could be predicted for large, water-filled channels, most porins form channels with weak selectivity for cations over anions (or vice versa). Data confirming this include zero current potential measurements (see Section 3.5, Fig. 5, and Table 4) and the behavior of chemically related solutes of different charge in liposome swelling assays (Nikaido and Rosenberg, 1983; Yoshimura and Nikaido, 1985). One notable exception to this rule is the protein P channel, which is almost anion specific (see Section 5.2).

The basis for the weak ion selectivity is charged amino acid side chains at the mouth of or within the channel, as indicated by two types of experiments. First, measurement of the relative permeability of anions and cations as a function of the pH of the salt solution bathing the membrane and within the pore has demonstrated that pores become increasingly cation selective with increasing pH (Benz *et al.*, 1979, 1984a). For example, with the anion-selective PhoE channel, major alterations in selectivity occurred between pH 3–5 and pH 7–9 (Fig. 6), levels at which amino acid side chain carboxyls and amines, respectively, might be expected to become deprotonated. A second indication of the role of charged amino acid chains in ion selectivity was provided by chemical modification of porins. Thus, amidation of the carboxyls of the cation-selective OmpF channel made the channel nonselective (Tokunaga *et al.*, 1981; Benz *et al.*, 1984b), whereas acetylation of amino groups (presumably on lysine side chains; Darveau *et al.*, 1984) in the anion-selective PhoE channel converted it to a cation-selective channel (Fig. 6). For the acetylated PhoE channel, it was demonstrated that titrating the channel between pH 3 and 5 still influenced the selectivity, but the influence of raising the pH from 7 to 9 on selectivity was lost upon acetylation of lysine amino groups (Fig. 6). These data strongly indicate the presence of charged amino acid side chains at the mouths of or within the channels of porins.

4.4 Porin Channels Are Usually Not Chemically Selective

Chemical selectivity could be broadly considered to involve a specific binding site for a given class of compounds. Only two of the 29 porins described in Table 1 (i.e., the LamB and protein P porins; see Section 5) have been shown to have specific substrate binding sites. This lack of chemical selectivity for most porins is emphasized here, since it contrasts dramatically with specific active transport and facilitated diffusion systems found in many biological membranes, including the bacterial cytoplasmic membrane. An important implication of this observation is that the diffusion of any given compound through a porin pore will obey Fick's law. Thus, in principle, the penetration rate of even an unknown compound through a porin channel can be predicted if we know its size and its charge relative to the selectivity of the porin (see, e.g., Fig. 5 of Nikaido and Rosenberg, 1981).

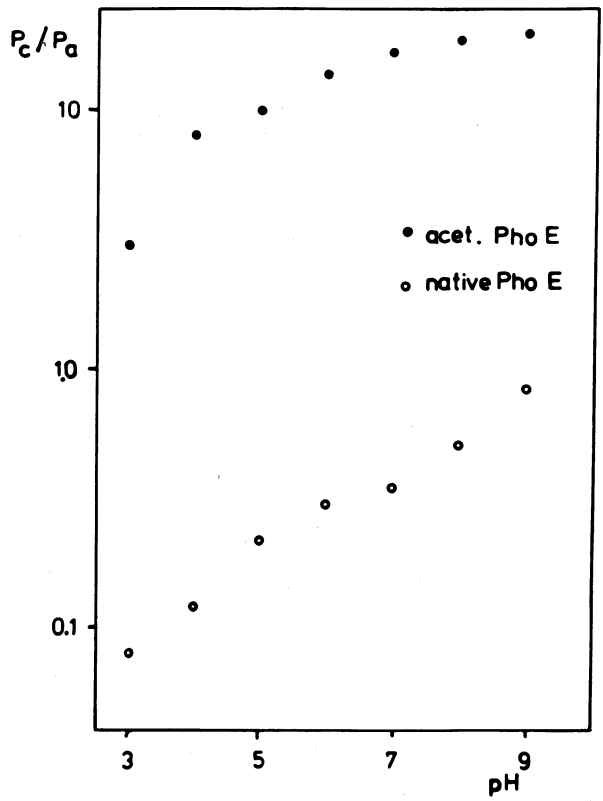

Figure 6. Influence of pH on the selectivity of native and acetylated PhoE porins. Each point on the graph was derived from experiments such as those shown in Figure 5. Note that the native PhoE channel is anion selective ($P_c/P_a < 1$), whereas the acetylated PhoE channel is cation selective ($P_c/P_a > 1$). While the selectivity of the native and acetylated PhoE E channels was similarly affected by altering the pH between 3 and 7, only the native PhoE channel was strongly affected by altering the pH between 7 and 9. This result can be best explained by the assumption that it is lysine side chains that were acetylated. (*Source*: Reprinted with permission from Darveau *et al.*, 1984.)

4.5 Porin Channels Are Usually Not Voltage Regulated

The research of Benz and colleagues (1978) on OmpF porin, now confirmed by other researchers, including myself, for a variety of porins, has demonstrated that porins are not voltage regulated or voltage gated. Thus, when membranes are preformed and porins allowed to self-incorporate into the membrane, as described in Section 3.4, the current through the porin channels is a linear function of the applied voltage, and the current-voltage curve passes through zero current at zero voltage (Fig. 7). In contrast, for a voltage-gated channel the current voltage curve would pass through the X axis (zero current)

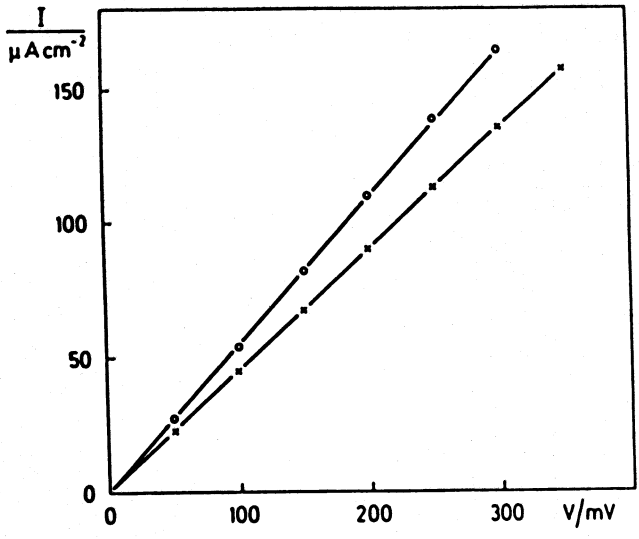

Figure 7. Linear relationship between applied voltage (V) and current (I) shown for two different membranes containing about 100 *E. coli* B OmpF porin channels. These data strongly argue that OmpF porin channels are not voltage regulated or voltage gated. (*Source*: Reprinted with permission from Benz *et al.*, 1978.)

at the gating (or "switch-on") voltage, whereas for a voltage-regulated channel the current-voltage relationship would deviate from linearity (due to a structural change in the pore-forming molecule). In principle, this lack of voltage gating has been confirmed by the demonstration of porin activity in liposomes (Tables 2 and 3) across the membranes of which no obvious voltage exists. In addition, Hellman and H. Nikaido (manuscript in preparation) have demonstrated no differences in the rates of penetration of cephaloridine through OmpF and OmpC channels when Donnan potentials varying between 5 mV and 80 mV were applied across the proteoliposome membrane.

In contrast, Schindler and Rosenbusch (1978, 1981), using a different reconstitution technique in black lipid bilayers, suggested that large voltages (approximately 100 mV) were required to switch on OmpF channels, while voltages greater than 150 mV caused closing of the porin channels. In view of these data and the rather modest Donnan potentials (20–30 mV) across *S. typhimurium* outer membranes (Stock *et al.*, 1977), we have proposed (Benz *et al.*, 1982) that reasons other than voltage gating of channels must be responsible for these effects. One possible reason is that the reconstitution technique of Schindler and Rosenbusch involves spreading of porin-containing vesicles on an air-water interface such that the vesicle lipids form a monolayer. The two monolayers on either side of the hole in the black lipid apparatus are then folded around to form a bilayer. Since porin molecules normally exist in a bilayer, it may be that, during the formation of a monolayer from the porin-

containing vesicles, the protein adopts an inappropriate configuration and thus does not penetrate the folded bilayer membrane. The high electric field could then facilitate the insertion of the porin into both monolayers of the bilayer, and this process could result in apparent opening of the pores.

The apparent closing of channels at higher voltages was observed for *Neisseria* porins (Young *et al.*, 1983); Lynch *et al.*, 1984) at voltages greater than 80 mV and for *Anabena variablis* porin at voltages greater than 50 mV (Benz and Böhme, 1985). In contrast, Benz and colleagues (1978) demonstrated that OmpF channels demonstrated linear current-voltage curves when millisecond voltage pulses up to 350 mV were applied (Fig. 6). The data for OmpF (Schindler and Rosenbusch, 1981) and *Neisseria* porins demonstrating voltage-dependent closing of channels can be rationalized by the observations (Benz *et al.*, 1978; Lynch *et al.*, 1984) that sustained application of high voltages caused a decreased channel lifetime such that the channels decayed under the influence of these voltages. Nevertheless, it must be stressed that it is extremely unlikely that potentials across the outer membrane of magnitudes equivalent to 80–150 mV are physiologically attainable.

4.6 Porins Are Often Functional as Trimeric Aggregates

Porin proteins are often present in their native state in the outer membrane as trimers (Table 1). This trimer state is usually maintained during purification even in the presence of strongly denaturing detergents such as SDS (Tokunaga *et al.*, 1979a,b,c). Recent data (Dorset *et al.*, 1984) on quasicrystalline arrays of the OmpF porin have suggested that an individual porin trimer contains three openings at the face of the porin trimer normally found on the surface of the outer membrane. These three channels coalesce into a single channel at or near the interface between the inner and outer monolayers of the outer membrane, and this single channel is what is normally exposed on the periplasmic side of the membrane. This model, although yet in its preliminary stages, implies that OmpF porin should not be functional as a monomeric unit, since the individual channels initiated on the outer face of the outer membrane do not penetrate the entire membrane. In agreement with this concept, Nakae and coworkers (Nakae *et al.*, 1979; Tokunaga *et al.*, 1979c) were unable to demonstrate pore-forming activity for the monomeric subunit of a variety of porins, including OmpF, even when the trimer was disaggregated by electrodialysis, a method that retained the β-sheet tertiary conformation of the porins. Nevertheless, a number of possible exceptions to the concept that porin trimers represent the functional units have been presented and are considered in more detail below.

Recent experiments by Zalman and Nikaido (1985) have suggested that the porin of *Paracoccus denitrificans* is a dimer. In the discussion of the same paper, it is also suggested that *Rhodopseudomonas* porins are dimers. The major evidence favoring dimers as the active species is chemical cross-linking, in which only a dimer species, but no trimer species, could be observed.

However, the generation of cross-linked oligomer species depends on a multitude of factors, including the availability of appropriately spaced and oriented amine groups. Under most conditions, far larger amounts of cross-linked dimer would be expected than of cross-linked trimer, even if the trimer were the native species (see Angus and Hancock, 1983, for a more complete discussion of cross-linking). A good example of the inherent danger of using cross-linking as the sole determinant of the number of subunits in a oligomer has been provided by the LamB protein, for which only the cross-linked dimer species was observed (Palva and Westerman, 1979; see also Palva and Randall, 1976, for similar data regarding the OmpF porin). Nevertheless, sedimentation equilibrium and X-ray diffraction data have strongly suggested that LamB is a trimer (Ishii *et al.*, 1981; Neuhaus, 1982). Therefore, the conclusions mentioned above concerning *P. denitrificans* and *Rhodopseudomonas* porins must be examined by alternative methods before it can be concluded with conviction that they represent an exception.

Many porins can be demonstrated on SDS polyacrylamide gels to run with lower mobility when solubilized at low temperatures (Table 1). Others, notably *P. aeruginosa* proteins F and D1 (Hancock and Carey, 1979) and *Neisseria gonorrhoeae* protein I (Wong and Johnston, 1981), do not form easily detectable SDS-stable oligomers on polyacrylamide gels. However, these data alone are not sufficient to allow the conclusion that the monomer forms of these proteins are the active species, since these proteins were usually purified in a detergent other than SDS. The combination of SDS treatment and the high electric field used in SDS polyacrylamide gel electrophoresis could induce the putative trimers to break down into monomers. Even in the case of *P. aeruginosa* protein F, which demonstrates function after SDS treatment (Yoshimura *et al.*, 1983), the conclusion that this protein functions as an monomer is complicated by the observation (Mutharia and Hancock, 1983; Hancock, 1985) that purified protein F preparations contain a small proportion of bands of lower electrophoretic mobility. The incidence of these putative protein F oligomer bands, which were identified as protein F on the basis of their reactivity with a protein F-specific monoclonal antibody and their 2-mercaptoethanol modifiability (Mutharia and Hancock, 1983), corresponds to the known low activity of protein F as a porin. Nevertheless, protein F and other porins described to be functional as monomers (e.g., Boehler-Kohler *et al.*, 1979; Weckesser *et al.*, 1984) must be examined in detail to see if the monomer units of porin trimers are capable of independent function as porins.

4.7 Porin Channels Are Not Influenced by Lipids and LPS

Black lipid bilayer experiments have demonstrated no influence of the lipid composition of the membranes on the single-channel conductance through OmpF (Benz *et al.*, 1978) porin channels. Thus, the usage of nonbacterial lipids such as oxidized cholesterol or monoolein instead of phospholipids did not influence any of the properties of the channel, although it did affect the

reconstitution rate (Benz *et al.*, 1978). Furthermore, the fluidity of the fatty acyl chains of phospholipids had no influence on the single-channel conductance through PhoE, based on the lack of effect of temperature between 10°C and 40°C on single-channel conductance (Benz *et al.*, 1984a). In addition, OmpF and PhoE porin channels showed the same single-channel conductance when negatively charged or neutral lipids constituted the membrane (Benz *et al.*, 1978; Benz *et al.*, 1984a). These data argue strongly that lipids do not influence the structure or function of porins, although caution should be used when applying these data to charged solutes of greater size.

In contrast, there is no real concensus on the requirement of lipopolysaccharide (LPS) for the reconstitution of functional porin channels. One of the problems is the difficulty of completely removing LPS from purified porin samples, since it strongly associates with porins during purification. Nevertheless, Schindler and Rosenbusch (1981) indicated that LPS-free OmpF porin was inactive in their black lipid bilayer reconstitution system and that LPS readdition would restore activity. We have been unable to reproduce this result using porin protein F of *P. aeruginosa* (R.P. Darveau and R.E.W. Hancock, unpublished), but we do not feel confident that we removed all of the LPS, since although it was absent by chemical tests, we could detect some LPS by ELISA using an LPS-specific monoclonal antibody. Yoshimura and coworkers (1983) observed no substantial effects of the addition of *P. aeruginosa* LPS in their reconstitution of protein F porin activity in proteoliposomes. However, again it was unclear that LPS was absent from the purified porin preparation.

To answer this question, we electroeluted *E. coli* OmpF and *P. aeruginosa* protein P trimers from SDS polyacrylamide gels. Using ELISA and LPS staining techniques, no LPS was found (the resolution limit was less than 0.01 moles of LPS per mole of porin). Despite the lack of LPS, no difference in the channel size or ion selectivity could be detected for electroeluted OmpF or protein P, compared to that of the conventionally purified porins (Parr *et al.*, 1986).

5. PORINS WITH UNUSUAL PROPERTIES

Up to this point, the "normal" properties of porin channels have been emphasized. A number of porin channels have now been demonstrated to have certain unique features. These are discussed briefly below.

5.1 LamB Porin: A Maltodextrin-Selective Channel

The LamB porin is produced by *E. coli* cells in response to maltose addition to the medium and is coregulated with a high-affinity maltose uptake system (for reviews, see Hennge and Boos, 1983; Schwartz, 1983). Early studies suggested that LamB protein formed a pore with a similar exclusion limit for saccharides to other *E. coli* porins (Nakae, 1979) and a slightly higher single-

TABLE 7. Specificity of LamB Porin-Containing Liposomes

Saccharide	Molecular Weight	Relative Rate of Permeation on a Per Weight Basis
Glucose	180	153
Sucrose	342	2.5
Lactose	342	9
Raffinose	504	0.1
Maltose	342	100
Maltotriose	504	97
Maltotetraose	666	37
Maltoheptaose	1152	8

Source: Data from Luckey and Nikaido (1980) adjusted to a maltose permeation rate of 100.

channel conductance in 1 M KCl (Boehler-Kohler *et al.*, 1979; Table 5). This was difficult to rationalize with in vivo observations that wild-type *E. coli* cells could grow on maltodextrins containing up to seven glucose residues (maltoheptose), whereas *lamB* mutants could only grow on maltodextrins with two or three glucose residues (Szmelcman *et al.*, 1976; Wandersman *et al.*, 1979).

This observation was in part rationalized by Luckey and Nikaido (1980), who demonstrated in liposome swelling assays that LamB-porin–containing liposomes were more permeable to maltodextrins, such as maltotetraose and maltotriose, than they were to the disaccharides sucrose and lactose (Table 7). Nakae and Ishii (1980) demonstrated similar results using proteoliposomes containing the enzyme amyloglucosidase. In agreement with this work, Ferenci and associates (1980) provided in vivo evidence from amylopectin-binding inhibition assays that LamB contained a relatively weak binding site for maltose and maltodextrins, although the affinity apparently increased with maltodextrin size. The previous observations that LamB porin formed channels somewhat larger (i.e., 2.7 nS in 1 M KCl) than those of other *E. coli* porins was demonstrated to be the result of the presence of another porin as a 1%–6% contaminant of all LamB preparations (Benz *et al.*, 1986). The LamB porin itself formed small (0.16 nS in 1M KCl) channels that could be completely blocked by maltose and maltodextrins, suggesting a maltodextrin binding site. In agreement with the in vivo data of Ferenci and associates (1980), the affinity of the channel increased with maltodextrin size, and a K_d (dissociation constant) of 4×10^{-4} M was measured for maltotriose binding.

5.2 Protein P: An Anion-Specific Channel

Under conditions of phosphate deficiency, *P. aeruginosa* cells are induced to produce a new major outer membrane protein P, presumably as a component of a high-affinity transport system (Hancock *et al.*, 1982). When protein P was purified and incorporated into black lipid bilayer membranes (Hancock *et al.*, 1982; Benz *et al.*, 1983), it gave rise to single-channel conductance events

significantly smaller than those of other porins studied to date (Table 4). In vivo measurements of permeability confirmed the apparent small channel size of protein P (Poole and Hancock, 1983).

Single-channel conductance measurements employing a variety of salt solutions indicated that the mobile species giving rise to the conductance was anions. For example, when the cation was large (e.g., $Tris^+$) and the anion small (Cl^-), a single-channel conductance similar to that for the salt K^+Cl^- was observed. In contrast, when the cation was small (K^+) and the anion large ($Hepes^-$), no conductance was observed (Hancock *et al.*, 1982). Zero current potential measurements confirmed that the protein P channel exhibited at least a 100-fold preference for anions over cations (Benz *et al.*, 1983). By varying the size of the permeating anion and fixing the type of cation (K^+), it was possible to obtain a quite accurate measurement of the effective diameter of the protein P channel (0.6 nm). A logarithmic relationship between the single-channel conductance and the anhydrous radius of the permeating anion (Benz *et al.*, 1983) indicated that the anions were at least partly dehydrated as they entered the channel. The removal of water from the anions would presumably require energy. The energy input for dehydration of anions could conceivably come from the binding of the anions to positively charged sites within the channel.

Three pieces of data obtained for the native protein P channel favored the existence of an anion-binding site or sites within the protein P channel. First, the anion selectivity of the channel could be explained by the existence of a positively charged cloud shell within the channel that could attract anions and repel cations. Second, titrating the pH of the salt solution bathing the channel between pH 7 and 9, thus causing potential deprotonation of lysine amino groups, caused a 2.5-fold decrease in single-channel anion conductance (Hancock *et al.*, 1983). Third, measurement of the single-channel conductance as a function of salt concentration for a variety of salts demonstrated saturation at high salt concentrations for the protein P channel but a linear relationship for all other porins studied (Fig. 8; Table 6). The data could be redrawn as an Eadie-Hofstee plot to demonstrate a binding site with K_ds for Cl^-, NO_3^-, $HCOO^-$, CH_3COO^-, and HCO_3^- of 40 mM, 30 mM, 300 mM, 80 mM, and 100 mM, respectively (Benz *et al.*, 1983; R. Benz, and R.E.W. Hancock, manuscript in preparation).

To investigate the actual nature of the anion-binding site in the protein P channel, we chemically acetylated available histidine and lysine amino groups with acetic anhydride (Hancock *et al.*, 1983). The protein remained in its trimeric configuration, although slight shifts in the apparent molecular weights of the monomer and trimer form were observed. Three major alterations in measured channel parameters were observed. First, the single-channel conductance in 1 M KCl decreased tenfold. Second, the anion selectivity, as measured by zero current potential measurements, decreased at least 30-fold, such that the acetylated channel was only three-fold selective for chloride over potassium. Third, single-channel conductance became a linear function of salt

concentration (Fig. 8), indicating that the strong ion-binding site had been lost and ions now diffused relatively freely through the channel.

These results strongly indicate that the basis of the salt concentration–dependent saturation and strong anion selectivity of the protein P channel is the presence of positively charged amino groups within the channel. Both the acetylation with acetic anhydride and the pH dependence of conductance favor the hypothesis that the ϵ-amino groups of lysines are involved.

More recently, a Tn501 insertion sequence mutant lacking protein P was isolated (Poole and Hancock, 1986). The mutant had altered phosphate transport kinetics, demonstrating a role for protein P in the inducible phosphate transport of *P. aeruginosa*. On the basis of this finding, we suspected that the anion-binding site of protein P should have a preference for phosphate over other anions. By measuring the blocking by phosphate of chloride flux through approximately 10,000 protein channels incorporated into a large lipid bilayer membrane, an inhibition constant K_i of 0.46 mM was determined (Hancock and Benz, 1986). This suggests that the anion-binding site can be described as a phosphate-binding site, since the binding affinity of the channel for phosphate is approximately two orders of magnitude higher than its binding affinity

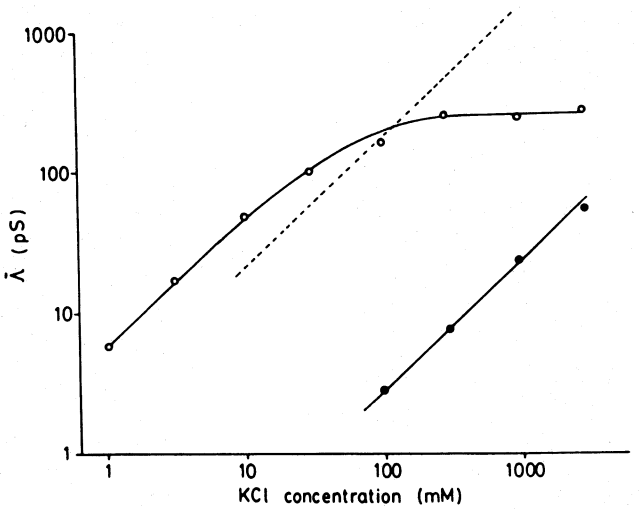

Figure 8. Dependence of the average single-channel conductance (Λ) on the concentration of KCl in the salt solution bathing the lipid bilayer membrane. Porins used were native protein P (open circles), acetylated protein P (closed circles), and native PhoE (dashed line). Single-channel conductances were obtained by averaging 100 or more stepwise conductance increases such as those seen in Figure 4. The saturation observed for native protein P can be explained by the presence of binding sites for chloride ions (K_d = 40 mM) within the protein P channel. The linear relationship observed for PhoE, acetylated protein P, and all other porins studied to date can be explained by relatively free movement of the ions through the channel, involving at most weak interactions with charged groups within the channel. (Reprinted with modifications with permission from Hancock *et al.*, 1983.)

for chloride. Chemical modification studies suggested that the phosphate-binding site of protein P involved charged lysine residues.

5.3 Protein F: A Large, Poorly Functional Channel

P. aeruginosa is an opportunistic pathogen that demonstrates high intrinsic resistance to most commonly used antibiotics. It has now been demonstrated that this property is largely due to the low outer membrane permeability of this organism (Angus *et al.*, 1982; Yoshimura and Nikaido, 1982; Nicas and Hancock, 1983). Studies using a mutant lacking a major outer membrane protein F have demonstrated that this protein constitutes the major porin in this cell (Nicas and Hancock, 1983). Nevertheless, it has been difficult to rationalize the high copy number (200,000 molecules of protein F per cell, Angus *et al.*, 11982) and apparent large channel size (Hancock *et al.*, 1979; Benz and Hancock, 1981; Yoshimura *et al.*, 1983; see Table 5 for summary) of protein F with the low outer membrane permeability of this organism. Three possibilities exist. One possibility is that the pore size of protein F was smaller than apparent in the three model system studies due to potential rearrangement during purification. We do not feel that this is true, since similar data were obtained whether purified protein F or outer membrane fragments were used for reconstitution experiments (Hancock and Nikaido, 1978; Hancock *et al.*, 1979; Benz and Hancock, 1981; Yoshimura, *et al.*, 1983). Furthermore, *P. aeruginosa* will grow on pentamethionine (Miller and Becker, 1978), whereas *E. coli* will grow on trimethionine and methionine-containing tetrapeptides but not on pentamethionine (Becker and Naider, 1974), a result thought to reflect the different channel sizes of the outer membranes of these organisms (Nikaido and Hancock, 1986).

A second possibility is that only a small percentage of protein F forms active functional channels. The low pore-forming ability of protein F in three model systems (Hancock *et al.*, 1979; Benz and Hancock 1981; Yoshimura *et al.*, 1983; summarized in part in Tables 2 and 3) is consistent with this possibility. Both in vivo and in vitro data suggest that less than 1% of protein F forms functional channels (Benz and Hancock, 1981; Nicas and Hancock, 1983). A third possibility is that the large channels previously observed for protein F preparations represent a contaminating porin protein present in small copy number (and responsible for the apparent large exclusion limit of the *P. aeruginosa*), whereas protein F itself forms only small channels. Such a possibility would be analogous to the situation now known to exist for the LamB protein (see Section 5.1). Further investigation is needed to distinguish these possibilities.

5.4. Porins from Nonbacterial Systems

Porinlike proteins have been demonstrated in a wide variety of nonbacterial systems. Since this chapter concerns bacterial porins, these nonbacterial porins

are only briefly discussed, and the reader is referred to a recent review by Benz (1985), who has summarized research on porinlike proteins from mitochondria and chloroplasts. Those mitochondrial porins studied to date form large (1.3–1.9 nm in diameter), water-filled, weakly anion-selective channels when reconstituted into model membrane systems (see, e.g., Colombini, 1979; Zalman *et al.*, 1980; Roos *et al.*, 1982; Benz, 1985). However, in contrast to bacterial porins (with the possible exception of *Anabena variablis* porin, see Section 4.5), mitochondrial porins are substantially voltage regulated, demonstrating significant reductions in single-channel conductance as a function of increasing applied voltage above 50 mV. The possibility that this voltage regulation is physiologically significant has been discussed (Fiek *et al.*, 1982). Another major difference of mitochondrial porins is that they insert asymetrically into lipid bilayer membranes and form asymmetric channels. For example, in one experiment, application of a voltage of +30 mV across the membrane resulted in a stable current of 14 $\mu A/cm^2$ through a group of mitochondrial porin channels, whereas application of a voltage of −30 mV (i.e., the same magnitude but opposite polarity) resulted in a current of −14 $\mu A/cm^2$, which decayed within 6 s to around −8 $\mu A/cm^2$. This behavior could be reproduced by cycling the transmembrane voltage between +30 mV and −30 mV. In contrast, the bacterial porins we have studied demonstrate no sidedness (asymmetry) in similar experiments.

6. IN VIVO DEMONSTRATIONS OF PORIN FUNCTION

Through the use of mutants and a variety of in vivo analysis systems developed by Zimmermann and Rosselet (1977), Sawai and colleagues (1977), and Nikaido and Rosenberg (1981), the properties of many of the porins described above have been confirmed in intact cells (Table 8). A number of excellent reviews have described these studies in depth (Nikaido and Nakae, 1979; Nikaido, 1979; Nakae, 1985; Nikaido and Vaara, 1985). The data show, with few exceptions, that results obtained in model membrane systems accurately mirror studies in intact cells (Table 8). This finding is of great importance, since it is much easier to create defined situations in reconstitution systems because the possible influence of other macromolecules can, by and large, be minimized or prevented.

7. CONCLUSIONS AND APPLICATIONS OF PORIN STUDIES TO OTHER SYSTEMS

The major conclusion one can derive from model system studies of bacterial outer membrane porins is the basic similarity of their properties. This suggests either a common evolutionary origin for bacterial porins or at least strong conservation through evolution of certain design principles. Thus, porins con-

TABLE 8. Comparison of in Vitro and in Vivo Demonstrations of Pore Properties of Bacterial Porins

Property	Porin	Reference to: Demonstration in Intact Cells	Reference to: Demonstration in Model System
Porin function	OmpF	Nikaido *et al.*, 1983	Benz *et al.*, 1979
	OmpC	Nikaido *et al.*, 1983	Nikaido and Rosenberg, 1983
	PhoE	Nikaido *et al.*, 1983	Nikaido and Rosenberg, 1983
	Protein K	Sutcliffe *et al.*, 1983	Whitfield *et al.*, 1983
	NmpC	Pugsley and Schnaitman, 1978a	Hindahl *et al.*, 1984
	LamB	Szmelcman *et al.*, 1976	Luckey and Nikaido, 1980
	F	Nicas and Hancock, 1983	Hancock *et al.*, 1979
	37kD	Sawai *et al.*, 1982	Kaneko *et al.*, 1984
	39–40kD	Sawai *et al.*, 1982	Kaneko *et al.*, 1984
Channel size	OmpF	Nikaido *et al.*, 1983	Nakae, 1976
	OmpC	Nikaido *et al.*, 1983	Benz *et al.*, 1982
	PhoE	Nikaido *et al.*, 1983	Benz *et al.*, 1982
Ion selectivity	OmpF	Nikaido *et al.*, 1983	Benz *et al.*, 1979
	OmpC	Nikaido *et al.*, 1983	Benz *et al.*, 1982
	PhoE	Nikaido *et al.*, 1983	Benz *et al.*, 1982
Maltodextrin selectivity	LamB	Ferenci *et al.*, 1980	Luckey and Nikaido, 1980
Poor function	F	Nicas and Hancock, 1983	Benz and Hancock, 1981

Note: OmpF, OmpC, PhoE, NmpC, and LamB are *E. coli* porins; F is a *P. aeruginosa* porin; 37kD and 39–40kD are *Klebsiella pneumoniae* porins. Similar data obtained for *S. typhimurium* porins have been omitted. Only sample references are presented; for a more complete listing, see Nakae, 1986.

stitute a set of proteins with higher similar physical properties (Table 1) that form large, water-filled channels in outer membrane bilayers (Tables 2–4). The properties of the water-filled channels of porins are dictated by defined physical principles (see Section 3) and by and large can be expressed in terms of a few simple statements (see Section 4).

Future studies of porins will be directed toward an understanding of the structure-function relationships of these proteins. To this end, the cloning and sequencing of the structural genes of many of these porins (see Appendix) and the initial crystallization studies of porins (see Chapter 7) have provided the preliminary requirements for such analyses. It should be possible in the next few years to obtain, through genetic, chemical, and physical manipulations of porin proteins, an accurate picture of the molecular anatomy of a porin channel. It is my opinion that channels such as the *E. coli* LamB and *P. aeruginosa* protein P porins, which have defined binding sites for maltodextrins and anions, respectively, provide an excellent opportunity for investigating the mechanism of facilitated diffusion, since these channels have substrate binding affinities equivalent to corresponding facilitated diffusion systems in red blood cells (Ho and Guidotti, 1975). Given the major advantages of these bacterial proteins, including ease of purification in high yields and the potential for genetic manipulation, they may well allow substantial advances in our understanding of facilitated diffusion systems. Already, studies with protein P are providing perhaps the clearest data on the mechanism of anion-specific channels. For example, examination of the conductance of protein P at low salt concentrations (Fig. 7) has demonstrated that, despite the constriction in this channel, the presence of an anion-binding site permits more rapid passage of anions than through the larger PhoE porin. Clearly, in further analyses of these proteins, model membrane studies will play a major role. In addition, the single-molecule sensitivity of the black lipid bilayer reconstitution system affords excellent potential for investigating the basis of the functional heterogeneity of individual porin protein molecules.

Another area in which studies of bacterial porins is having a substantial impact is the development and sophistication of model membrane systems. The reasonable simplicity of bacterial porins as a model system has permitted the refinement and more complete definition of the liposome swelling assay (Nikaido and Rosenberg, 1983) and the establishment of a set of base data for the black lipid bilayer system (Benz *et al.*, 1979, 1983). These developments have resulted in an enormous explosion of interest recently in porins in a wide variety of important bacteria and mitochondria, and in recent investigations on chloroplast porin (Flügge and Benz, 1984) and lens gap junction protein (Nikaido and Rosenberg, 1985).

A further potential area of importance for porin studies is the refinement of the channel theories discussed in Section 3. For example, although Nikaido and Rosenberg (1983) expressed doubts concerning the application of the Renkin corrections to small biological channels such as porins, it is clear that the agreement of their calculations, based on these corrections, with inde-

pendent estimates of channel size (Table 5) argues strongly for the relevance of such mathematical treatment of porin channel data.

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