PORIN FROM BACTERIAL AND MITOCHONDRIAL OUTER MEMBRANES

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I. INTRODUCTION

The cell envelope of gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium* consists of three different layers: the outer membrane, the peptidoglycan layer, and the inner membrane (Figure 1). The inner membrane acts as a real diffusion barrier and it contains besides the respiration chain a large number of different transport systems for substrates. The peptidoglycan (or murein) layer protects the bacteria from osmotic lysis. The outer membrane is a molecular filter with defined exclusion limits for hydrophilic substrates. The active components of the molecular sieving properties of the outer membrane are a major class of proteins called "porins".[1] The porins usually have a molecular weight between 30,000 and 50,000 daltons and they form trimers in the outer membrane which are in many cases tightly associated with the peptidoglycan layer. Porin trimers were isolated from the outer membrane of a variety of different gram-negative organisms and their properties were studied in reconstitution experiments with lipid vesicles and artificial lipid bilayer membranes. Furthermore, protein sequence analysis and genetic manipulations were performed on the three major porins of *E. coli* and the three-dimensional structure of one of them was analyzed.

The origin of the first eukaryotic cell is still puzzling. The endosymbiotic theory proposes that the symbiosis of two or three prokaryotic cells resulted in the first eukaryotic cell. According to this theory gram-negative bacteria should be the ancestors of mitochondria and chloroplasts. Both cell organelles have in agreement with the endosymbiotic theory outer membranes which contain pores. The pores of the outer mitochondrial membrane and the outer membrane of the chloroplast envelope share some properties with the bacterial porins, in particular, the large size. On the other hand, they appear to be voltage controlled and they are processed by the cell and not by the genetic instrumentation of the mitochondria or the chloroplasts.

In the past 10 years a large number of reviews have been written on the function,[2-4] structure,[5-8] genetics,[9,10] and biogenesis[11-13] of outer membranes of gram-negative bacteria. Furthermore, reviews have been published on specific components of the outer membranes, like lipopolysaccharides[14-16] and lipoprotein.[17] The topic of this review is another major component of the outer membrane, the porins. This review will also focus on the properties of the mitochondrial porin and it will give a brief description of the recently described chloroplast pore or porin.[18]

II. BACTERIAL PORINS

A. Structure and Composition of the Outer Membrane of Gram-Negative Bacteria

Structure and composition of the outer membrane of gram-negative bacteria have been

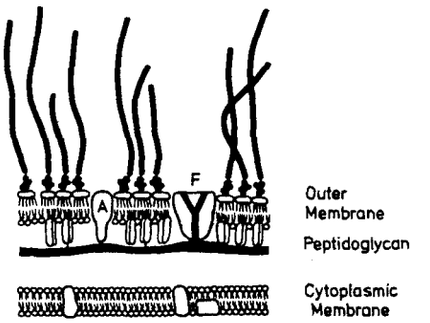


FIGURE 1. Structure of the cell envelope of enteric bacteria. (A) Omp A; (F) Omp F trimers with the pore in cross-section.

discussed in detail in a number of excellent recent reviews.[4,8,11] Here only the basic features of the outer membrane are given that are needed for the understanding of the function of the porins. The cytoplasmic membrane of gram-negative and most other bacteria is supported on the outside by a large macromolecule which protects the bacteria from osmotic lysis. This peptidoglycan or murein layer consists of a network of amino sugars and amino acids.[19] The amino sugars (*N*-acetylglucosaminyl-*N*-acetylmuramyl dimers) form long linear strands which are covalently linked together between two muramyl residues by short tetrapeptides.[19] The cell envelope of gram-negative bacteria also contains an outer membrane. Components of this membrane are either covalently linked to or they interact via ion bridges with the peptidoglycan layer.[8] The outer membrane shows the typical trilaminar structure in osmicated, positively stained thin sections under the electron microscope.[20] The periplasmic space between inner and outer membrane occupies a certain fraction of the total cell volume.[21] It appears to be isoosmotic with the cytoplasm, which means that the turgor pressure (approximately 3.5 bar[8]) is maintained across the outer membrane attached to the peptidoglycan layer. The periplasmic space is strongly anionic as compared with the external medium.[21]

1. Isolation of the Outer Membrane

The disruption of gram-negative bacteria followed by differential centrifugation leads to a cell envelope fraction which is composed of outer membrane, cytoplasmatic membrane, and peptidoglycan layers.[22,23] The first successful separation of the outer membrane from the other components of the cell envelope consisted of a sucrose density gradient centrifugation[24,25] following a preparation of an envelope fraction from spheroplasts.[26] The spheroplasts were obtained from cells treated with lysozyme in the presence of EDTA. The cytoplasmic membrane could be separated from the outer membrane because of the higher density of the latter which may be caused by the larger content of carbohydrate of the outer membrane.[27] The first isolation procedure presented by Miura and Mizushima[25] was followed by many other procedures. The first step is always the disruption of the cells. This can be done by a French pressure cell treatment, by ultrasonication, by shaking the cells in the presence of glass beads, or by osmotic lysis of the spheroplasts. The outer membrane can be separated from the crude envelope fraction by the above-mentioned sucrose density gradient centrifugation and modifications of it.[27-29] Another method uses the relatively low solubility of the outer membrane in nonionic detergents. The cytoplasmic membrane is removed in this case by a washing procedure with Triton® X-100 in the presence of Mg^{++}.[30] The ionic detergent sodium lauryl sarcosinate can be used for the complete solubilization of the cytoplasmatic membrane in the absence of Mg^{++}.[31] It has to be noted, however, that in both cases a considerable fraction of phospholipids and lipopolysaccharides may be lost from the outer membrane during the washing procedure with detergents.[30]

Outer and inner membranes of gram-negative bacteria differ in charge because of the large number of negatively charged groups attached to the lipopolysaccharides. Using this difference in negative charges outer membrane and cytoplasmatic membrane may be separated using free-flow[32] or sucrose-gradient electrophoresis.[33] On the other hand, the large excess of negatively charged groups makes the outer membrane more hydrophilic than the cytoplasmic membrane. This has been used to separate outer and inner membranes by a phenyl-Sepharose CL-4B column[34] or by aqueous-phase separation in the presence of polyethylene glycol (mol wt 20,000) and phosphate.[34] The outer membrane can be eluted from the column or in the latter case is fractionated into the more hydrophilic bottom phase of the aqueous-phase separation.

2. Lipids

The outer membrane of gram-negative bacteria contains lipids, lipopolysaccharides (LPS), and proteins as major components. The lipid composition of the outer membrane is very similar to that of the inner membrane. This similarity in the lipid composition most probably has to do with the in vivo synthesis of the outer membrane. Several possibilities are discussed. The first possibility consists in formation of vesicles containing lipids, LPS, and protein at the inner membranes. These vesicles are released, diffuse across the periplasmic space, and fuse with the outer membrane.[8] Electron microscopic studies provide some evidence for close contacts between the cytoplasmic membrane and the outer membrane. These "adhesion sites" could be structures where inner and outer membranes are fused, thus allowing diffusion of newly synthesized membrane components from the inner to the outer membrane.[8] These "adhesion sites" could also be composed of inverted micelles which have been discussed as allowing the rapid flip-flop of lipid molecules across membranes.[35] Similar contact sites have also been discussed as existing between outer and inner membrane of mitochondria.[36,37] All possibilities mentioned above would result in a similar lipid composition of the inner and the outer membrane and allow us to understand why the outer membrane of cyanobacteria contains carotenoids.[34]

The major lipid of the enteric bacteria *E. coli* and *S. typhimurium* is the zwitterionic phosphatidylethanolamine.[38] Besides this neutral lipid the outer membrane of both organisms also contains small amounts of the negatively charged phospholipids phosphatidylglycerol and cardiolipin.[39] The fatty acid residues of the phospholipids contain mostly C-18 chains. The number of double bonds in the side chains varies with the growth temperature. Some of the fatty acid residues contain cyclopropane rings instead of double bonds to increase the membrane fluidity.[38,40] In enteric bacteria, the phospholipids are exclusively located in the inner monolayer of the outer membrane where they cover about 50% of the surface.[41] The rest is covered by proteins.[42] Nonenteric bacteria like *Neisseria gonorrhoeae* or the cyanobacteria also contain phospholipids in the outer monolayer of the outer membrane.[4,34]

3. Lipopolysaccharides (LPS)

The outer surface of enteric bacteria is covered by about 40% LPS and 60% protein. LPS are amphipatic molecules which show a structural similarity to lipids. Common to all LPS is the hydrophobic lipid A while the hydrophilic polysaccharide moiety (*O*-antigen) may vary within a single species.[14-16] The basic structure of the lipid A is a D-glucosaminyl-β-D-glucosamine backbone to which between five and seven side chains are linked via ester and amide bonds.[43] The fatty acid residues have a chain length between 14 and 16 carbon atoms and are normally unsaturated.[44] They contain very often hydroxyl groups in the 3-position which can be used for the binding of further fatty acid residues via ester bonds.[14,42,43] In enteric bacteria, the lipopolysaccharides form a strong barrier for the diffusion of hydrophobic molecules through the outer membrane.[44,45] This is partly due to the long oligosaccharide side chain attached to the lipid A. Mutants with drastically reduced length of the oligosaccharides ("rough" mutants) very often have an increased permeability of the outer

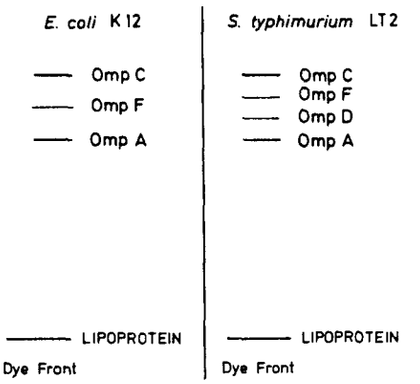


FIGURE 2. SDS-polyacrylamide gel pattern of outer membrane proteins of *E. coli* K12[48] (a) and *S. typhimurium* LT2.[49] The protein samples were heated in SDS to 100°C before electrophoresis.

membrane for hydrophobic compounds.[44] On the other hand, this low permeability may also be caused by the close association of the LPS molecules in the outer monolayer via van der Waals interactions between the side chains[45] and via ion bridges between charged groups[46] located at the polysaccharide moiety.

4. Proteins

The protein pattern of the outer membrane of gram-negative bacteria is relatively simple. Electrophoretograms show only a limited number of bands which correspond to "major" proteins (Figure 2). Nevertheless, there existed in the past 15 years some confusion about the number of bands and the molecular weight of the corresponding proteins in studies performed in different laboratories on *E. coli* outer membranes.[50-54] This was caused by the peculiar aggregation behavior of the outer membrane proteins and the differences in the preparation of the protein samples for the electrophoresis. It is now clear that the outer membrane proteins of *E. coli* and *S. typhimurium* have to be boiled for several minutes in a solution which contains at least 1% sodium dodecyl sulfate (SDS). Outer membrane proteins of *P. aeruginosa* have to be kept at 100°C for an even longer time to receive reproducible results in the SDS-polyacrylamide electrophoresis.[55]

There also existed a considerable confusion in the nomenclature of the outer membrane proteins of *E. coli* because of the use of several different nomenclature systems by different laboratories.[48,54,56-60] Reeves[9] proposed a uniform nomenclature system for the outer membrane proteins of *E. coli* and *S. typhimurium* which is now widely accepted. In this system, the protein or gene product is named after its structural gene; i.e., OmpA is the product of the ompA gene (Omp stands for outer membrane protein). Table 1 contains the molecular weights of the outer membrane proteins of *E. coli* and *S. typhimurium* and a comparison of the nomenclatures used by the different laboratories.

5. Lipoprotein (Braun's Lipoprotein)

The lipoprotein (Braun's lipoprotein) was the first outer membrane protein of *E. coli*, which has been isolated and purified.[72] It is a small protein with 58 amino acids and a molecular weight of 7,200.[73-75] Part of the protein is covalently bound to the peptidoglycan layer (2.4 to 10^5 copies per cell[17]). The free lipoprotein comprises about two thirds of the total protein.[76] Both forms have the same composition and contain a large proportion of α-helical regions,[77] which are not commonly found among the outer membrane proteins (see below). The lipoprotein gene has been cloned and its DNA sequence has been determined.[78]

Table 1
NOMENCLATURE AND MOLECULAR WEIGHT OF THE MAJOR OUTER MEMBRANE PROTEIN OF *E. COLI* K-12 AND OF *S. TYPHIMURIUM* LT2

E. coli

Uniform nomenclature[9]	Mol wt	Schngitman[56,57]	Henning[58,59]	Lugtenberg[48,60]	Mizushima[54]	Foulds[61]
OmpA	35,200[62,63]	3a	II*	d	010	TolG
OmpF	37,100[64,65]	Ia	Ia	b	09	—
OmpC	38,300[66]	Ib	Ib	c	08	—
PhoE	36,800[67]	Nmp AB protein	Ic	e	—	E

S. typhimurium

Uniform nomenclature[9]	Mol wt	Ames[49]	Nakae[69,70]
OmpA	35,600[68]	33 k	—
OmpF	39,300[69]	35 k	39 k
OmpC	39,800[69]	36 k	40 k
OmpD	38,000[69]	34 k	38 k
PhoE	(34,000)[71]	—	—

The precursor form has a 20-amino-acid signal peptide at its amino-terminal end. Mutations of the lipoprotein and the lack of lipoprotein in *E. coli* mutants result in changes of the envelope structure and the cellular morphology.[79] These results suggest that the lipoprotein plays an important role in structure and stability of the outer membrane. Lipoproteins are present in all *Enterobacteriaceae*,[78,80-82] with well-preserved structures and a number of other gram-negative bacteria,[83,84] while others (like *Neisseria gonorrhoeae*[85]) contain no lipoprotein.

6. Heat-Modifiable Protein

The OmpA protein in outer membrane of *Enterobacteriaceae* is also known as a heat-modifiable protein because of the change of its apparent molecular weight after heating of the protein samples in SDS.[52,86,87] At room temperature the protein has an apparent molecular weight of about 25,000. If the protein is heated it runs on a SDS-polyacrylamide gel with an apparent molecular weight of around 35,000. The OmpA protein of *E. coli* K-12 has 325 amino acid residues with a total molecular weight of about 35,000.[62,63] The amino acid sequence is not particularly hydrophobicity. OmpA genes from different enteric bacteria have been cloned and expressed in *E. coli*. The primary structures of the gene products have been deduced from the genetic code.[68,88-90] The primary sequences of the different proteins show a close analogy. OmpA protein from *E. coli* and from *Enterobacter aerogenes* have for instance a homology of 85%.[90] In contrast to lipoprotein, the OmpA protein of *E. coli* has a high content of β-sheet structure.[62,91] The high content of β-sheet structure is presumably responsible for the influence of heating on the apparent molecular weight because SDS binds in a larger concentration to the unheated protein. Based on the observation that OmpA acts as a receptor for phages[92] and colicins[93] similar to the porins, it is possible that it could form a pore in the outer membrane. However, reconstitution experiments with lipid bilayer membranes in the presence of OmpA did not give any evidence for pore formation.[94] On the other hand, there exists a certain local,[95] but not general,[62] homology between the porins (OmpF, OmpC, and PhoE) and the OmpA protein of *E. coli*. This analogy has either to do with the evolution or with the processing and assembly of these proteins.[95] The role of the 10^5 copies of OmpA in the outer membrane is still not clear. OmpA-deficient mutants show an increase of other components of the outer membrane[96,97] (porins, lipid, LPS), but have still a reasonable growth rate. Similar to the lipoprotein, the OmpA protein seems to have a structural function in the outer membrane.

7. Porins

Another class of major outer membrane proteins are the porins also called "peptidoglycan-associated proteins",[4] "peptidoglycan-associated general diffusion pore proteins",[8] or "matrix proteins".[98] They are usually tightly associated through ion bridges with the peptidoglycan layer. Furthermore, they form diffusion channels in the outer membrane,[1,90-101] which led to the name "porin" first proposed by Nakae.[1] The name "matrix protein" is somewhat misleading because the real matrix proteins of the outer membrane are presumably the lipoprotein and OmpA. Furthermore, the hexagonal structure which resulted in this definition[98] is only observed in *E. coli B* under rather artificial conditions. The name "peptidoglycan-associated proteins" does not give any information on the function of this major class of outer membrane proteins. Here, the name porin will be used throughout for them especially because some of the porins may not be tightly associated with the peptidoglycan layer. The outer membranes contain up to 10^5 copies of the different porins per cell.[98]

Porins, tightly associated with the peptidoglycan layer, can easily be isolated by the method proposed by Rosenbusch.[98] After breaking the cells, the cell envelope is solubilized in 2% SDS at 60°C. The unsoluble material contains the peptidoglycan with the covalently bound lipoprotein and the associated porins. The porin can either be separated from the peptidoglycan by digestion of the peptidoglycan layer using lysozyme and trypsin or in a more elegant way by a salt extraction method.[87,102,103] Boiling of the porin-peptidoglycan

complex for 5 min in SDS leads also to dissociation but it denatures the protein.[87,98] Porins from *E. coli*,[58] *S. typhimurium*,[87] and a number of other gram-negative bacteria[55,104-107] have been isolated and characterized. The proteins have molecular weights, ranging between 30,000 and 50,000. They are organized in the outer membrane as trimers of three identical subunits.[102,108,109] The amino acid composition of many porins is known at present.[69,98] It is obviously not particularly hydrophobic. Furthermore, the protein sequence of three porins of *E. coli* has been determined by sequencing either the protein directly or the corresponding structural gene.[64-67] The longest hydrophobic part of the OmpF sequence comprises about 10 hydrophobic amino acids. The large number of charged amino acids are presumably used to stabilize the tertiary and quaternary structure of the porin trimers which contain a large amount of β-sheet structure[98] via internal ion bridges.

Whereas the outer membrane of *E. coli* B contains under normal growth conditions only one porin[98] (OmpF), two porins, OmpF and OmpC, are present in the outer membrane of *E. coli* K-12.[58,59,111] Their expression is influenced by the osmolarity of the culture medium.[112,113] OmpF is preferentially expressed if the cells grow in media of low osmolarity or high cAMP levels.[114] On the other hand, OmpC is preferentially expressed in media of high osmolarity. The genes involved in the regulation of the osmolarity-sensitive expression of both porins are known.[115,116] Nevertheless, it is not clear how the cells sense the osmolarity of their environment. The OmpF and OmpC have approximately the same exclusion limit for the diffusion of hydrophilic solutes[117] (molecular weight around 650) and are otherwise so closely related that it is difficult to understand why one porin is expressed at low osmolarity and the other one at high osmolarity of the culture media. Furthermore, cells with chimera genes where a reciprocal exchange of the promoter-signal sequence regions has been constructed,[118] show normal growth rates. This indicates that the synthesis of one or both porins under different conditions may not be critical for cell physiology.

The outer membrane of *S. typhimurium* contains under normal growth conditions three different porins — OmpF, OmpC, and OmpD — with apparent molecular weights of 35,000, 36,000, and 34,000, respectively.[49,69] The *Salmonella* porins are closely related to one another and to the corresponding *E. coli* porins and show immunological cross-reaction.[119] They have a somewhat larger effective diameter than the *E. coli* porins because they are permeable for hydrophilic substrates with an upper limit around 800.[101] The largest exclusion limit has been found for the outer membrane of *Pseudomonas aeruginosa*. This membrane is permeable for hydrophilic substrates up to a molecular weight around 5000.[120,121] Responsible for this large exclusion limit is the presence of the protein F which forms trimers in the outer membrane.[109] *P. aereuginosa* is, on the other hand, quite resistant to antibiotics. From this it has been concluded that only a limited number of pores are open at a given time,[122] a conclusion which has been confirmed in vesicle permeability measurements[123,124] and artificial bilayer experiments[125] in the presence of protein F.

A number of porins are induced in the outer membrane of gram-negative bacteria if the cells grow under special conditions. Phosphate limitation leads in *E. coli* to the expression of a number of different proteins.[125] Among them is an alkaline phosphatase, and the porin PhoE.[125] This porin is weakly anion selective, whereas the OmpF and OmpC pores are cation selective.[126] Based on its role in the phosphate uptake it has been suggested that the PhoE pore contains a weak binding site for phosphate.[127,128] Porin PhoE shows a close homology to the porins OmpF and OmpC of *E. coli*.[66,67,129] Phosphate limitation induces also in the outer membrane of *P. aeruginosa* an additional porin channel.[130] The protein P trimers[109] form a highly anion-selective channel with a binding site for anions.[131] The apparent cross-section of the P-porin of *P. aeruginosa* is much smaller than that of the PhoE porin of *E. coli*.[126] The same may also be valid for a comparison between the protein P pore and the recently discovered PhoE porin of *S. typhimurium* outer membrane.[71] Besides the above-mentioned three different but closely related porins, the outer membrane of *E. coli* can also contain two to three other porins which share some common features with the above-described

porins. Two of them appear either in revertants of porin-deficient *E. coli* mutants (NmpC)[132] or in prophage PA2 stable mutants (Lc).[133] Both porins seem to be closely related. The third additional porin, the porin K, was found in encapsulated *E. coli* strains[134] and has presumably something to do with the capsule because it is normally absent in the nonencapsulated strains. The porin K shows some analogy to the OmpF porin.[135,136] Most porins from *E. coli*, and from *S. typhimurium*, have been identified as receptors for phages.

The growth of *E. coli* on maltose or maltodextrin-containing media leads to the induction of another outer membrane protein, the LamB porin.[137] This protein (mol wt 47,400; 421 amino acids)[138] is the product of the lamB gene and it is also known as maltoporin. Although the LamB porin also forms trimers in the outer membrane,[139] it is quite different from the other porins and shows no immunological cross-reactions with them.[119] The LamB protein acts as the receptor of the phage lambda and forms in vitro (and presumably also in vivo) tight complexes with the phage tail.[140-142] It is only loosely associated with the peptidoglycan layer.[143] Reconstitution experiments with lipid vesicles show that the presence of LamB porin increases the permeability of the vesicles for maltose and some maltodextrins much more than for sucrose.[144] This result has been explained by the assumption of a specific but most probably weak binding site for maltose inside the pore. A similar protein to LamB has also been described to exist in the outer membrane of *S. typhimurium*,[145] whereas a glucose inducible porin (D_1) was found in *P. aeruginosa*.[146] This protein also forms trimers in the outer membrane.[109]

8. Minor Proteins

The outer membrane of gram-negative bacteria contains also a large number of so-called "minor proteins". Some of them have been identified to be involved in the uptake of iron[147] or of vitamin B_{12}.[148] On the other hand, the enzymatic activity of the outer membrane of *E. coli* is extremely low and no ATPase activity or electron transfer-type reaction has been detected so far. This means that it is rather unlikely that the cell can create or control a potential difference across the outer membrane as it does across the inner membrane. Nevertheless, there exists some kind of Donnan-equilibrium potential across the outer membrane of *E. coli* and *S. typhimurium* which is under normal growth conditions on the order of 30 mV and which does not create a considerable asymmetry in the ion distribution across the outer membrane.[19]

B. Permeability Properties of the Outer Membrane of Gram-Negative Bacteria

1. The Hydrophobic Barrier

The permeability and physiology of the outer membrane of gram-negative bacteria will be described in detail in a forthcoming article by T. Nakae in *CRC Critical Reviews in Microbiology*. Therefore, only the basic features of the permeability properties of the outer membrane are given here. In enteric bacteria, the outer membrane acts as a diffusion barrier for hydrophobic and hydrophilic molecules. Hydrophobic molecules normally cross (i.e., in the absence of a specific transport pathway) membranes by a diffusion process. The permeability coefficient for this process is given by:

$$P = \frac{\gamma \cdot D}{d} \quad (1)$$

where γ is the partition coefficient of the hydrophobic substance between membrane and aqueous phase, D is the diffusion coefficient inside the membrane, and d is the thickness of the membrane. The permeability of a membrane can be regulated by either decreasing the product $\gamma \cdot D$ in Equation 1 or by decreasing the concentration of the hydrophobic compounds on the aqueous side of the membrane-water interface. In fact, the enteric bacteria have a hydrophilic surface which is caused by the long sugar chains of the LPS molecules.[149]

In deep rough mutants the length of the sugar chains is reduced and LPS of the outer monolayer is partially replaced by phospholipids.[29] The outer membrane of these mutants has an increased permeability for hydrophobic molecules. The same has been observed in EDTA-treated cells where part of the LPS is released[151] and replaced by phospholipid coming either from the inner membrane or the inner monolayer of the outer membrane.[152] On the other hand, the tight aggregation of the LPS molecules in the outer membrane and their saturated side chains could represent a diffusion barrier. The incorporation of phospholipids in this barrier could disturb its order and thus result in an increased permeability. Nonenteric bacteria like *Neisseria gonorrhoeae* which do not live in the environment of bile salts and long-chain fatty acids presumably also contain phospholipids in the outer monolayer of the outer membrane.[4] Their outer membranes represent, in agreement with the above-described role of LPS in enteric bacteria, a lower barrier for the permeation of hydrophobic molecules.[153]

2. Permeation of Hydrophilic Molecules

In vivo studies with plasmolyzed cells of *E. coli* and *S. typhimurium* have shown that the outer membrane has a clear size limit for the penetration of hydrophilic substances.[120,154] The outer membrane is fully permeable for sucrose (mol wt 342) and raffinose (mol wt 504), whereas stachyose (mol wt 666) penetrates only partially into the periplasmic space. Furthermore, the outer membrane is virtually impermeable to hydrophilic solutes of molecular weight greater than 1000. In the case of *E. coli*, there exists a sharp exclusion limit around mol wt 600 to 650 for the penetration of sugars through the outer membrane,[120] which has also been observed for the penetration of peptides.[155] Reconstitution experiments with vesicles in the presence of "major" outer membrane proteins have shown that one to three protein bands are responsible for the penetration of hydrophilic compounds through the outer membrane of *E. coli* and *S. typhimurium*.[1,99-107,156] Mutants of *S. typhimurium* which were deficient in the major outer membrane proteins showed in agreement with this a reduced permeability towards cephaloridin, a hydrophilic solute of mol wt 415.[157]

There exist two possibilities for the penetration of hydrophilic compounds through the outer membrane of gram-negative bacteria. One is the diffusion through large water-filled pores and the other possibility is the facilitated diffusion by a carrier mechanism. The facilitated diffusion mechanism has been ruled out by the extremely low specificity of most porin proteins (except LamB, PhoE, and Protein P) and the low activation energy of the membrane permeability.[120] Thus, it is very likely that most porins form a general diffusion pathway for hydrophilic substrates in the outer membrane of gram-negative bacteria, i.e., a water-filled pore. It has to be noted, however, that porin-free mutants also show a reasonable growth rate under laboratory conditions where all nutrients are present at high concentrations.[157] But this result is consistent with the finding that the pores in the outer membrane become rate limiting only at low substrate conditions.[4] This has to be considered as natural and there exists no evidence that an appreciable fraction of the up to 10^5 porin pores of e.g., *E. coli* could be closed under physiological conditions.[4]

The size (i.e., the exclusion molecular weight) of the pores in the outer membrane of a given gram-negative bacteria is typical for this organism. *E. coli* and *S. typhimurium* outer membranes are permeable for hydrophilic solutes up to mol wt 600 to 800,[1,101] whereas solutes up to a molecular weight of about 5000 can penetrate the outer membrane of *P. aeruginosa*.[121] There exists some evidence that the exclusion molecular weight for the penetration of the outer membrane of certain "exotic" gram-negative bacteria could be as large as 20,000.[158]

C. Biosynthesis of Porins

Figure 3 shows the location of the genes involved in the synthesis of the major outer membrane proteins in *E. coli*. Common to all of them is the synthesis of the proteins in a larger precursor form with a leader sequence at the N-terminal end. The leader sequence

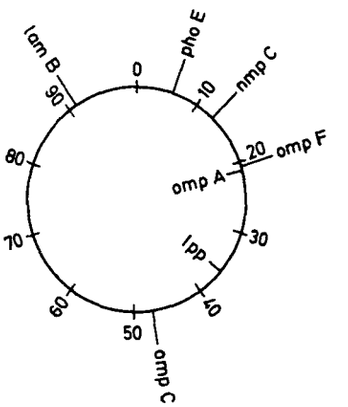


FIGURE 3. Location of the genes of major outer membrane proteins and of the porins on the *E. coli* chromosome.

contains between 21 and 22 amino acids in the case of the three porins OmpF,[65] OmpC,[66] and PhoE,[67] whereas OmpA[63,159] and Braun's lipoprotein[160] have leader sequences of 21 and 20 amino acids, respectively. The existence of leader sequences with approximately the same length suggests that the major outer membrane proteins may share a common pathway for their biosynthesis. In fact, there exist some indications that this is true for OmpA and the porins.[95] The mechanism of synthesis and processing of the lipoprotein studied in detail by Inouye and co-workers appears to be somewhat different (for a review see Reference 11). Lipoprotein is presumably synthesized at membrane bound polyribosomes. The first synthesized leader sequence combines with the membrane.[161] After processing of the polypeptide extension by the membrane bound prolipoprotein leader peptidase,[162] the peptide elongates during the synthesis through the cytoplasmic membrane. The protein may fold in the periplasmic space and may reach its mature form in the outer membrane. The periplasmic form of the lipoprotein is still sensitive to trypsin. The mechanism of insertion into the outer membrane is still unknown for the lipoprotein and for the other outer membrane proteins.

Zimmermann and Wickner[164] studied the biosynthesis of OmpA. OmpA protein is synthesized at free ribosomes in the cell as a water-soluble precursor which accumulates inside the cell if the membrane potential is partially inhibited by the addition of an uncoupler of the oxidative phosphorylation.[164] At normal membrane potential, the pro-OmpA protein is translocated through the cytoplasmic membrane and processed by a membrane-bound leader peptidase. This leader peptidase[165] cleaves also pro-membrane proteins of the cytoplasmic membrane and proteins secreted into the periplasmic space,[166] but it is not identical to the leader peptidase which processes the lipoprotein.[163] So far, it is not clear in all cases if the pro-porins follow the same mechanism as the pro-OmpA, i.e., if a membrane potential across the cytoplasmic membrane is needed for the translocation of the pro-porins across this membrane. However, also in the case of the OmpF-porin it is likely that the chain is almost, if not entirely, complete before the processing starts.[167] This result provides some evidence that the pro-OmpF is also synthesized at free ribosomes and translocated after completion. When inner and outer membrane are separated, the pro-OmpF is localized in the inner membrane, which indicates its processing at the inner membrane by a membrane-bound leader peptidase.[167] This leader peptidase would be identical to that which cleaves the polypeptide extension of pro-OmpA. The processing of the pro-OmpF into the mature protein occurs very rapidly with an apparent lifetime of the precursor of 30 sec.[167] The processed porin may undergo some folding in the periplasmic space. The mechanism of the assembly of the trimers in the outer membrane from three mature monomers is still completely unknown, although there exists some indication for a somewhat delayed formation of stable

trimers from metastable trimers in the case of LamB.[163] It has to be mentioned, however, that the translocation and assembly of pro-porin in phenethyl alcohol treated cells contradicts somewhat the above described (tentative) pathway.[168] Lower concentrations of phenethyl alcohol inhibited the processing and assembly of pro-porin, but pro-porin has also been found to be loosely associated with the outer membrane.[168] On the other hand, there also exists some evidence for a co-translational processing of periplasmic proteins in *E. coli*, i.e., translocation of the nascent chain after completion of approximately 80% of its entire length.[110]

D. Isolation and Purification of Porin Trimers

Common to most porins is the tight but non-covalent association with the peptidoglycan layer. This tight association can be used for the isolation and purification of porin trimers. The outer membranes of many gram-negative bacteria contain more than one single porin. Here it is best to use mutants which are available for *E. coli* and *S. typhimurium*. On the other hand, it is also possible to repress the expression of one or several porin species. Well-known examples for this are the discoveries of the PhoE porin of *E. coli* K-12[111,125] and of the porin P[130] of *P. aeruginosa*. Both porins are expressed in cells growing under phosphate limitations.[125,130] PhoE at least partially replaces in the *E. coli* outer membrane the "normal" porins OmpF and OmpC under these conditions.[125]

1. Porins from E. coli and *S. typhimurium*

The isolation and purification of porins from *E. coli* and *S. typhimurium* is based on the tight association between porin trimers and peptidoglycan layer. Earlier published procedures for the isolation were based on the enzymatic degradation of the peptidoglycan using trypsin and lysozyme.[1,99-101] This method has the disadvantage that the porin was not obtained in individual trimers but in aggregates of large molecular weight. A more recent method uses a salt extraction method for the preparation of trimers first proposed by Nakamura and Mizushima.[87] The method was extended by Tokunaga et al.[102] and a recent excellent description has been given by Nikaido.[103] A short scheme of the procedure is as follows: the gram-negative bacteria are grown under normal culture conditions at 37°C; the addition of 0.5% glucose represses the expression of the maltoporin (LamB); after washing the cells they are resuspended in a small volume and passed three times through a French pressure cell; the cell envelope containing the cytoplasmic membrane, the peptidoglycan, and the outer membrane are pelleted by centrifugation. The SDS-soluble components of the outer and the inner membrane are removed by washing the pellet with a buffer solution containing 2% SDS at elevated temperature (up to 60°C). After centrifugation the pellet should contain only peptidoglycan and the associated porin. Considerable amounts of non-porin proteins in the pellet can be removed by a repetition of the SDS-wash step followed by centrifugation. The peptidoglycan-associated porin preparation is resuspended in a buffer which contains as essential components, besides 1% SDS, 0.4 *M* NaCl and 5 m*M* EDTA.[102,103] The supernatant of a subsequent centrifugation step is applied to a Sepharose 4B or a Sephacryl® S-200 superfine column. The porin trimers are eluted from the column with the same buffer. The SDS content of the protein solution can be decreased by a dialysis procedure, but this is not essential for the pore-forming activity which remains constant for several months at 4°C.[103] The protein solution can also be lyophilized without any loss of the pore-forming activity.[169] In lyophilized form the protein remains active for at least 1 year stored in a freezer at −20°C.

Maltoporin from *E. coli* can be isolated using the interaction between the LamB protein and the maltose-binding protein (MBP) as has been proposed by Nikaido and co-workers.[103,170] This method decreases the amount of other porins in the preparation which may obscure the permeability properties of the maltoporin.[144] Cells are grown in a maltose-containing medium which causes maximum induction of the LamB protein. The protein is

extracted from the outer membrane of cells grown in the late exponential phase using a buffer which contains 2% Triton® X-100 as detergent and 10 m*M* EDTA. The supernatent of a subsequent centrifugation step is applied to a MBP-Sepharose column. After washing the column, the maltoporin trimers are eluted with a buffer solution containing 0.2 *M* NaCl and 0.1% Triton® X-100. The pore forming activity is also in this case stable for several months if the maltoporin is stored at 4°C.[103]

2. Porins from P. aeruginosa and Other Gram-Negative Bacteria

Three different porins have been isolated from the *P. aeruginosa* outer membrane by Hancock, Nikaido and co-workers: the protein F,[121,123] the glucose inducible protein D1,[146] and the protein P induced by phosphate limitation.[130] The outer membrane of *P. aeruginosa* is very sensitive to EDTA, which leads to a degradation of the outer membrane and to an unsatisfactory separation between inner and outer membranes.[121] Therefore, alternative methods have been proposed which avoid the use of EDTA and which are based on sucrose density centrifugation.[55,121] These methods may also be of advantage in the case of other gram-negative bacteria,[106] where the outer membrane is susceptible to EDTA and where the porins are only loosely associated with the peptidoglycan layer that the above described method for *E. coli* and *S. typhimurium* cannot be used. The method described in the following was established by Hancock and Nikaido.[121]

P. aeruginosa cells are grown on normal culture medium for the isolation of protein F. The cells are harvested, washed, and passed twice through a French press. Lysozyme is then added to the cell envelope fraction to degrade the peptidoglycan. A crude purification is obtained by a sucrose density centrifugation using 70 and 15% sucrose. The upper part of the gradient is applied to a second gradient containing four steps of 70, 64, 58, and 52% sucrose.[121] Four bands are obtained after an overnight centrifugation. The two bottom bands OM1 and OM2 are outer membrane fractions where only OM2 contains a small contamination of inner membrane. The outer membrane contains four major protein bands corresponding to apparent molecular weights between 10,000 and 40,000.[121,171] Some of them are heat modifiable.[55] The protein F has an apparent molecular weight of 36,000.[123] It can be purified after solubilization of the outer membrane in Triton® X-100 and EDTA followed by ion exchange chromatography[123] or in cholate followed by gel filtration on a Sephacryl®-S200 column.[124] In a more recent publication, it has been observed that the pore-forming activity of protein F is not destroyed by the use of dodecyl sulfate as detergent.[124] Thus, a differential extraction of the cell envelope has been proposed[124] which is very similar to that described above for the isolation and purification of porins of *E. coli* and *S. typhimurium*.

For the isolation and purification of the glucose-inducible protein D1 of *P. aeruginosa*, outer membranes have been prepared from glucose-grown cells without addition of lysozyme.[146] Otherwise the procedure was the same as described above. The outer membrane is suspended in a buffer solution containing 2% Triton® X-100 to remove the Triton®-soluble part of the outer membrane. The pellet of the subsequent centrifugation is resuspended in a buffer containing besides 2% Triton® X-100 10 m*M* EDTA to remove protein D1 from the peptidoglycan. The supernatant of a centrifugation step is applied to a DEAE Sephacel® column.[146] The porin is eluted with column buffers containing increasing concentrations of NaCl.

Protein P is induced in the *P. aeruginosa* outer membrane if the cells grow on phosphate-deficient medium[130] ($c_{P_i} \leq 0.2$ mM). This porin has properties which are unique among all porins. The isolation procedure for protein P follows the procedure described above for the glucose-inducible protein D1,[55,146] with the exception that only one sucrose density gradient centrifugation is used for the isolation of the outer membrane from the cell envelope. Cell envelopes in 20% sucrose are layered onto a step gradient with 70% sucrose in the bottom and 60% sucrose in the top layer. The bottom layer contains after centrifugation the outer membrane.[130] Then the outer membrane is solubilized by Triton® X-100-EDTA treatment

following a Triton® X-100 washing step. Protein P and contaminating protein F elute in the same fractions of the DEAE Sephacel® column.[130] These fractions are concentrated and mixed with excess (2% SDS) and applied to a Sepharose 4B column where protein P elutes just after the void volume.[130]

A sucrose density gradient centrifugation has also been used for the isolation of the outer membrane of *Neisseria gonorrhoeae* from the cell envelope.[104] The cells grown on agar plates are harvested, washed, and ruptured. The cell envelope obtained as a pellet in a centrifugation step is resuspended and fractionated on a linear 20 to 70% sucrose gradient centrifugation.[104] The lower band contains the purified outer membranes. The porin (protein I) is obtained by a cholate extraction of the outer membrane at high ionic strength followed by high pressure liquid chromatography (HPLC).[104] Protein I from *N. gonorrhoeae* has also been isolated using a different isolation procedure.[172] This method uses the positively charged detergent hexadecyltrimethylammonium bromide for the extraction of whole cells and the solubilization of the precipitate with 1 *M* $CaCl_2$.[172] It is not described in further detail here because the purification procedure is based on the use of organic solvents like ethanol and acetone which may destroy the pore-forming activity of most porins. The same applies to the extraction of the outer membrane with 50% acetic acid, which has been used for the isolation of a porin from *Proteus mirabilis* outer membrane.[105]

E. Reconstitution of Porin into Liposomes

1. Evaluation of Exclusive Molecular Weights of the Diffusion Through Porin Pores

In vitro systems have several advantages over in vivo systems. They are relatively simple, contain only a few components, and allow a good control of the conditions. The main disadvantage of in vitro systems is, on the other hand, the possibility of artifacts. Many of the properties of the outer membrane of gram-negative bacteria have been studied in reconstitution experiments with liposomes or lipid vesicles.[1,99-103] These experiments have been used to identify the pore-forming proteins of the outer membrane.[1,99,121,123] Furthermore, the size of the pores has been measured in penetration studies with radiolabeled solutes.[1,99,121] The basic principle of these measurements is the following.[1,99,103] Liposomes are formed in a buffer solution containing two radiolabeled solutes from lipids and lipopolysaccharides in the presence of pure protein or protein fractions. The liposomes are obtained by shaking the suspension followed by mild sonication in bath-type sonicator. Lipids and LPS have been used in a molar ratio of 8:1, although LPS may not be essential for the formation of protein-containing vesicles.[103]

Both radiolabeled solutes are entrapped in the liposomes during the liposome formation process. One of them could be [^{3}H] dextran with a large molecular weight such that it does not penetrate the pores. The other solute of lower molecular weight should be labeled with ^{14}C. The liposomes are subsequently passed through a Sepharose 4B column where the liposomes elute just after the void volume. The radiolabeled solutes outside the liposomes are retarded on the column. If the lower-molecular-weight solute is permeable through the pores it will diffuse out through the pores during the elution process and the original ^{14}C to ^{3}H ratio is drastically reduced. Measured as a function of the molecular weight of the [^{14}C] solute, the ^{14}C to ^{3}H ratio will allow the evaluation of the exclusion molecular weight of the pore.[1,99,103] Such measurements have been performed with porins isolated from *E. coli*,[1] *S. typhimurium*,[99,101,156] and *P. aeruginosa*.[121,123,124] The exclusion molecular weights of the porin pores of *E. coli* and *S. typhimurium* is about mol wt 600 and 800, respectively. This indicates that the diameter of the *S. typhimurium* pores could be a little larger than that of *E. coli*, which is about 1.1 nm. The diameter of the pores of *P. aeruginosa* outer membrane appears to be much larger, and hydrophilic solutes with a molecular weight up to 5000 can penetrate the pores[121,123,124] (see Figure 4). This result is consistent with in vivo studies,[120] although the permeability of *P. aeruginosa* outer membrane for large hydrophilic solutes has recently been questioned.[173]

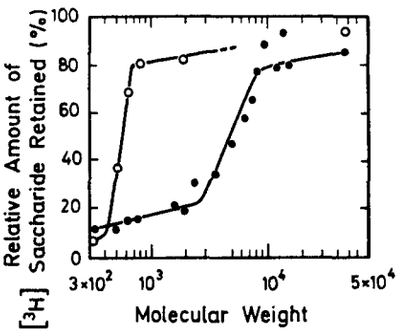


FIGURE 4. Exclusion limit for saccharides in reconstituted outer membrane vesicles of *P. aeruginosa* PA01 (closed circles) and *S. typhimurium* LT2 M1 (open circles). (From Hancock, R. E. W. and Nikaido, H., *J. Bacteriol.*, 136, 381, 1978. With permission.)

The use of the above-described method may lead to artifactual results if the permeability of only one solute is measured. Denaturated membrane protein may adsorb and incorporate into liposomes and lipid vesicles without the formation of a defined permeatron pathway. This undefined pathway could still be permeable for ions and large uncharged solutes like sucrose. However, the existence of a sharp, defined exclusion molecular weight for the penetration of solutes is extremely unlikely in this case. The exclusion limit of such a pathway may be smeared out across a large range of molecular weights.

2. Swelling of Porin-Containing Liposomes

The above-described method for the evaluation of the exclusion molecular weight does not allow measurement of the penetration kinetics of hydrophilic solutes through the porin pores. The eluation of the liposomes from the Sepharose 4B column needs such a long time that only all-or-nothing processes can be measured. Thus, a possible specificity of the permeability pathways cannot be detected. To overcome this problem and to measure specificities of porin pores and penetration kinetics Nikaido and co-workers[103,106,117,124,144,174] modified the liposome swelling assay established by Bangham and co-workers.[175,176] Liposomes are formed from phospholipids and porin in a buffer which contains a certain concentration of dextran impermeable through the porin pores. LPS has not been used in this assay and the phospholipids could either be isolated from *E. coli* or commercially available phospholipids could be used. The liposomes are added under rapid mixing to an isotonic solution of a test solute. If this solute can penetrate the porin pores, the total concentration of solutes inside the liposomes increases and the liposomes swell because of the influx of water driven by the osmotic gradient across the liposome membranes. This swelling process can be detected by a decrease of the average refractive index of the liposomes, i.e., of the optical density. The initial swelling rate can be used as a measure for the penetration rate of the test solute through the porin pores if the movement of the test solute and not the water permeability of the liposome membrane is rate limiting. Using the same liposome preparation the penetration rates of different solutes can be compared. The logarithm of the relative rate of permeation through an unspecific pore is a linear function of the molecular weights of the solutes[144,174] (see also Figure 5). The application of the theoretical considerations of Renkin[182] to the relative rates of permeation allow an estimation of the channel size.[144,174]

Luckey and Nikaido[144] using this method could show that maltoporin (LamB) of *E. coli* outer membrane has a certain specificity for maltose and maltodextrins as compared with other saccharides. This finding is consistent with in vivo studies where it has been shown

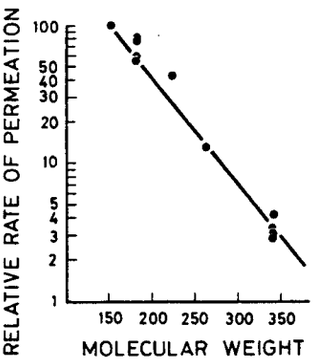


FIGURE 5. Relative rates of permeation of different sugars into phospholipid-porin vesicles. The sugars were (from top to bottom): mol wt 150, L-arabinose; mol wt 180, D-galactose, D-fructose, D-mannose, and D-glucose; mol wt 221, *N*-acetyl-D-glucosamine; mol wt 262, 2,3 diacetamido-2,3-dideoxy-D-glucose; mol wt 342, sucrose, melibiose, maltose, and lactose. (From Nikaido, H. and Rosenberg, E. Y., *J. Gen. Physiol.*, 77, 121, 1981. By copyright permission of the Rockerfeller University Press.)

that the maltoporin facilitated the uptake of maltose and maltotriose.[137] On the other hand, the observed specificity seems to contradict the results obtained from the determination of the exclusion molecular weight performed with the maltoporin, where no specificity has been observed.[177] This is, however, easy to understand because the latter method cannot be used to measure permeation rates of different solutes.

The liposome swelling assay has also been used to determine and to compare the size of different porin pores.[103,106,117,174] In experiments with liposomes in the presence of the three different porins (OmpF, OmpC, and PhoE) of *E. coli* outer membrane, it has been found that all three pores have approximately the same size.[117] The effective diameter of the OmpF pore (1.2 nm) appears only a little larger than that of the other two pores (1.1 nm).[117] Positively charged solutes have smaller penetration rates through the PhoE pore than negatively charged solutes. This indicates a preference for the penetration of negatively charged solutes through the PhoE pore, whereas only a poor specificity has been observed for the OmpF and OmpC pores.[117] These observations are in agreement with the role of the PhoE pore in phosphate uptake in phosphate-starved cells.[60,125,178,179] The effective pore diameter of porins from *E. coli* and *Rhodopseudomonas sphaeroides* is about 1.2 nm,[106,117] whereas that of the porin F of *P. aeruginosa* is about 2.2 nm.[124]

The penetration rates through the porin channels have also been measured as a function of temperature.[174] The Q_{10} rate of diffusion of *N*-acetyl-D-glucosamine was about 1.25,[174] which corresponds to an activation energy of about 14 kJ/mol (3.3 kcal/mol). This activation energy is very similar to that of the diffusion process in the aqueous phase which has activation energies between 10 and 16 kJ/mol (corresponding to 3 to 4 kcal/mol). This means that the diffusion of solutes through a porin pore is very similar to that in the aqueous phase, which supports the view that the porin trimers form usually wide water-filled channels. Diffusion processes through the lipid core of membranes have in contrast to the diffusion through the porin pores much larger activation energies between 40 and 100 kJ/mol (10 to 25 kcal/mol).[180,181]

3. The Vesicle Permeability Assay

Nakae and co-workers[183] have proposed an alternative way for the measurement of penetration rates through porin pores. This method is based on the earlier observation that the

periplasmic β-lactamase activity can be used for the study of the penetration of the outer membrane.[184] Enzymes like alkaline phosphatase or α-glucosidase are trapped in porin-containing unilamellar vesicles during the formation process. The activity of the intravesicular enzyme is directly related to the influx of substrates, provided that the affinity of the enzyme is the same for all substrates.[183] The permeation of different solutes through the pores is measured via the products of the enzymes. This method has been used to measure the permeability of porin- and maltoporin-containing vesicles in the presence of maltose, maltotriose, and raffinose.[183,185] The results do not indicate such a strong specificity of the maltoporin for maltose and maltotriose as has been found in the liposome swelling assay.[144] The reason for this discrepancy is not clear at present. It cannot be excluded, on the other hand, that LamB acts as a general diffusion pathway and as a facilitated diffusion unit for maltose and maltodextrins. It has to be kept in mind, however, that the enzymes inside the vesicles create an additional concentration gradient across the vesicle membranes. This means that solutes and their degradation products (moving in opposite directions) compete for the same pore, thus lowering the influx of the solutes into the vesicles.

The properties of chemically modified OmpF porin of *E. coli* outer membrane have also been studied in the vesicle permeability assay described above.[186] Acetylation and succinylation modify amino groups of the porin trimers, whereas amidation blocks negatively charged groups.[187] The results of the vesicle permeability assay have been found to be in qualitative agreement with the expected influence of free charges on the movement of charged solutes through the OmpF pore.[186] The decrease of negatively charged groups on the surface of the protein or inside the pore leads to an increase of the permeability of negatively charged solutes, and vice versa. The experimental results give no indication that the chemical modification leads to conformational changes of the trimers.

F. Properties of Porin Pores in Artificial Lipid Bilayer Membranes

Experiments with reconstituted vesicles provide excellent information about the presence and the size of the pores formed by a porin. More detailed information about the pore interior and the pore selectivity can be obtained from experiments with solvent-containing[188] and solvent-free[189] lipid bilayer membranes. The reconstitution of the porins into lipid bilayers and the properties of the porin channels from *E. coli*, *S. typhimurium*, and *Ps. aeruginosa* in lipid bilayer membranes will be described in the following. The porins of gram-negative bacteria form in general large water-filled pores with a poor selectivity for ions in lipid bilayer membranes. However, exceptions also exist. Protein P from *Ps. aeruginosa* outer membrane forms highly anion-selective channels in lipid bilayer membranes.

1. Reconstitution of Porins into Planar Lipid Bilayers

Three different methods have been used successfully to reconstitute porins into lipid bilayer membranes. First, detergent-solubilized porin is directly added to the aqueous phase bathing a membrane;[169,190] second, the lipid bilayer membrane is formed from reconstituted vesicles according to the Montal-Mueller method;[190-192] and third, the porin is inserted into the planar bilayer via fusion of the reconstituted vesicles with the membrane.[193]

The simplest method consists of the addition of purified porin from a stock solution containing 0.1% dodecyl sulfate or Triton® X-100 to the aqueous phase bathing a black lipid bilayer membrane.[169]

Figure 6 shows such an experiment. Porin OmpC of *S. typhimurium* outer membrane was added in a final concentration of 100 ng/mℓ to a black membrane of egg phosphatidylcholine/*n*-decane. After an initial lag of 4 min, presumably due to diffusion of the protein through unstirred layers, the conductance increased by about three orders of magnitude within about 30 min. Only a slight additional increase (as compared with the initial one) occurred after that time.

Since in all the experiments with porins a steady conductance level could not be reached,

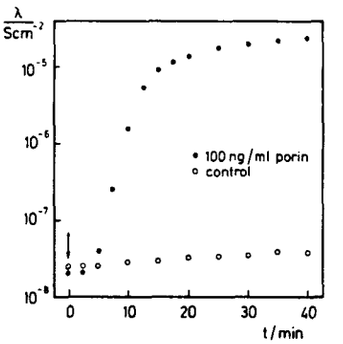


FIGURE 6. Specific membrane conductance as a function of time after addition of 100 ng/mℓ OmpC trimers of *S. typhimurium* to a black membrane from egg phosphatidylcholine/*n*-decane (closed circles, arrow). The open circles represent a control experiment in which only 10 μg/mℓ SDS was added to another membrane; 1*M* KCl was present in the aqueous phase.

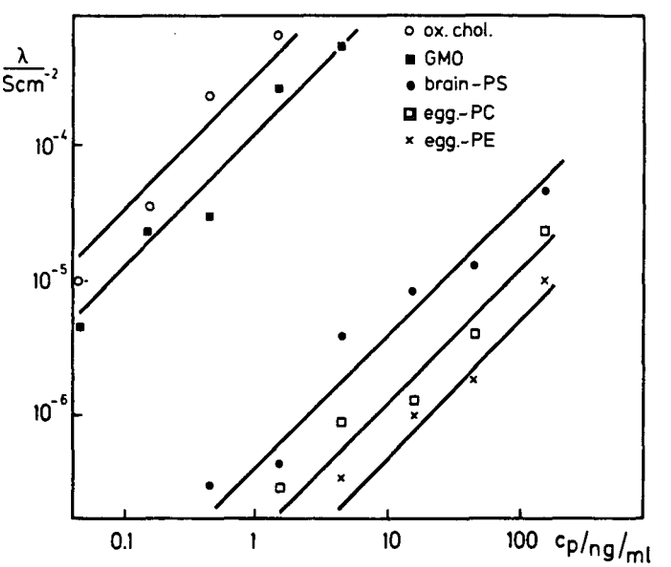


FIGURE 7. Specific membrane conductance λ as a function of the concentration of porins of *S. typhimurium* (OmpF, OmpC, and OmpD) in the aqueous phase. The membranes were formed from different lipids in *n*-decane. The aqueous phase contained 1 *M* KCl and less than 5 μg/mℓ SDS.

the dependence of the conductance on various parameters was difficult to determine. A meaningful comparison was possible, however, on the basis of experiments similar to those presented in Figure 6, using the conductance value at a fixed time after addition of the protein. Figure 7 shows the influence of the membrane composition on the incorporation of the porins from *S. typhimurium* strain TA 1014 (OmpF, OmpC, and OmpD) into the membranes. For membranes made from oxidized cholesterol or monoolein, the conductance is about two to three orders of magnitude larger than for membranes made from phospholipids. A similar "lipid specificity" was also found for the porins from *E. coli* and *P. aeruginosa*. The strong conductance increase indicates the incorporation of many conductive units into the lipid bilayer membranes. This allows the investigation of the influence of the voltage

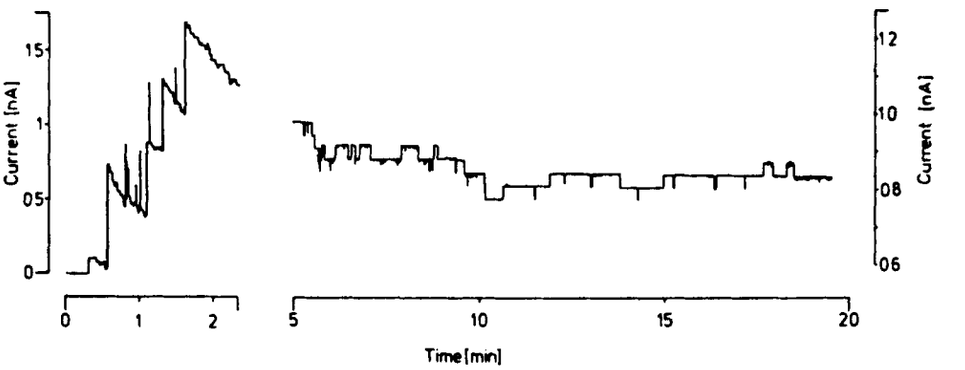


FIGURE 8. Initiation of conductance in a porin-containing membrane made from monolayers. The left side shows large current jumps which appear at 240 mV membrane potential in 0.1 *M* NaCl. Each large increment was followed by a decay in small steps (0.14 nS) as shown on the right side of the figure. (From Schlinder, H. and Rosenbusch, P., *Proc. Natl. Acad. Sci. U.S.A.*, 75, 3751, 1978. With permission.)

on the porin pores. The observed current was a linear function of the applied voltage up to 150 mV for porins of *E. coli*,[169] *S. typhimurium*,[70] and *P. aeruginosa*,[130,131,194] whereas the membrane current decreased in the presence of a porin (protein I) of *N. gonorrhoeae* outer membrane, even if this porin was added to the aqueous phase.[190]

Schindler and Rosenbusch[191,192] used an alternative method for incorporation of porin molecules into bilayer membranes. Reconstituted vesicles from lipids and porin were added to the aqueous phases on both sides of a thin Teflon® foil which has a small circular hole (100 to 200 μm diameter) above the initial water level. Monolayers form now on the surfaces of the two aqueous compartments. It has been suggested that these monolayers have the same composition as the vesicles.[191] The water levels on both sides of the foil are now raised above the hole and a folded membrane is formed across the hole. No pores are observed in the membranes immediately after formation at low membrane voltages. Membrane potentials around 100 mV are needed to induce the pores in steps much larger than one single conductive unit. Figure 8 shows such an experiment taken from Schindler and Rosenbusch.[191] The initial jumps indicate the formation of pore multimers, whereas the following decay is due to inactivation of the single conductive unit caused by the high voltage.

The control of the membrane permeability by voltage-gated pores in the outer membrane of gram-negative bacteria is difficult to understand. The channel density in the outer membrane of gram-negative bacteria is about 10^{12} pores per square centimeter,[4] and the time constant of the membrane is very small under normal conditions.[70] Asymmetry potentials cannot exist for a long time across the outer membrane and Donnan-potentials are by far too small to reach 100 mV.[21] The physiological role of voltage-gated pores in the outer membrane is obscure so far and other reasons may be responsible for the observed voltage effects as has been discussed in detail elsewhere.[195,196]

The third possibility of incorporation of porin molecules into planar lipid bilayer membranes is through fusion of reconstituted vesicles with planar bilayers.[193] The vesicles are added to the cis side of the membrane. Upon addition of calcium ions to the cis side and establishing an osmotic gradient hyperosmotic at the cis side, the fusion process begins as indicated by a step-wise increase of the membrane current. The fusion process stops after removal of the osmotic gradient by addition of the osmotic active substance to the trans side. Furthermore, the influx of water into the reconstituted vesicles increases the yield of fusion without any osmotic gradient across the membrane.[193] It is interesting to note that the fusion process resulted immediately in open pores in the lipid bilayer membranes and that no voltage induced activation of pores has been observed. Only a minor inactivation has been found at high voltages prior to membrane breakage.[193]

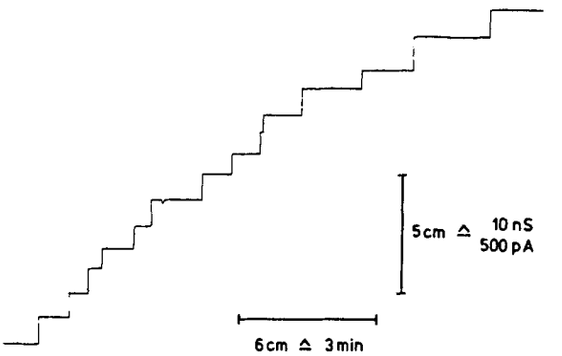


FIGURE 9. Step-wise increase of the membrane current in the presence of 1 ng/mℓ OmpF of *E. coli* K 12 added to the aqueous phase containing 1 *M* KCl. The membrane was formed from egg-phosphatidylcholine/*n*-decane; V_m = 50 mV.

2. Single Channel Analysis

The addition of small amounts (1 to 10 ng/mℓ) of porins to the aqueous phase bathing a membrane of small surface resulted in a step-wise increase of the membrane current at a given voltage. These current jumps were not observed when only the detergents dodecylsulfate or Triton® X-100 were added to the aqueous phase in the same concentration as in the presence of porin. Figure 9 shows an experiment in the presence of porin OmpF from *E. coli*. As can be seen from Figure 9, all steps were directed upwards, whereas downward steps were only rarely observed. Similar results are also found for the porins from *S. typhimurium*[70] and *P. aeruginosa*.[131,194] The lifetime of all porin pores was at least 1 min as could be seen from records extending over long times. Only pores observed in the presence of total outer membrane from *P. aeruginosa* had a much shorter lifetime (on the order of 50 to 100 ms), although the absolute level of the pore conductance was not changed as compared with purified porin F.[194]

The conductance steps which have been observed with the different porins were not uniform in size but were distributed over a certain conductance range (compare Figure 9). The reason for this broad distribution is not clear. It has to be noted, however, that broad distributions have been observed for all conditions, i.e., different detergents[169] and also for porin activity from osmotic shock fluids.[197] Only the anion channel formed by porin P of *P. aeruginosa* outer membrane gave a narrow distribution of the single conductance steps.[198]

For all porins described in this review (except porin P from *P. aeruginosa*[131,198]) the average pore conductance $\bar{\Lambda}$ was a linear function of the specific conductance σ of the aqueous phase; i.e., the ratio of $\bar{\Lambda}$ to σ varied only little in contrast to variations of $\bar{\Lambda}$ by two orders of magnitude (see Table 2). This is also reflected in Figure 10 where the average pore conductance $\bar{\Lambda}$ for the F-porin of *P. aeruginosa* is given as a function of σ. The data points could be fitted with a straight line. The same is valid for the porins from *S. typhimurium*[70] and *E. coli*[199] (dotted line in Figure 10), although the ratio of $\bar{\Lambda}$ to σ is larger for *P. aeruginosa* porin F. This can be explained by the larger effective diameter of the *P. aeruginosa* pore,[194] which is also consistent with the liposome permeability assay using carbohydrates of different sizes[121] and the liposome swelling method.[124] It is interesting to note that even large organic cations and anions like $Tris^+$, $N(CH_2CH_3)_4^+$, and $Hepes^-$ were able to pass through the porin pores with little or no interaction with the pore interior.

The linear relationship between specific aqueous conductance σ and the single channel conductance $\bar{\Lambda}$ given for most porin pores suggests that the ions move inside the pore similar as in an aqueous environment. This finding allows a rough estimate of the effective size of the pores. Assuming that the porin pores are filled with a solution of the same specific

Table 2
SINGLE CHANNEL CONDUCTANCE $\bar{\Lambda}$ OF THE OmpC PORE OF *S. TYPHIMURIUM* IN DIFFERENT SALT SOLUTIONS[70]

Salt	c/M	$\bar{\Lambda}$/nS	$\bar{\Lambda}/\sigma/10^{-8}$ cm
LiCl	1.0	0.9	1.3
NaCl	1.0	1.8	2.1
KCl	1.0	2.4	2.1
$MgCl_2$	0.5	0.62	1.0
K_2SO_4	0.5	1.4	1.8
$MgSO_4$	0.5	0.32	1.0
$Tris^+$ $Hepes^-$	0.5	0.088	1.2
$N(C_2H_5)^+$ $Hepes^-$	0.5	0.045	0.94

Note: σ is the specific conductance of the corresponding salt solution.

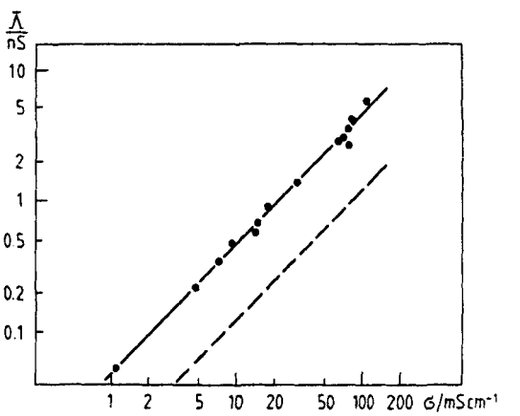


FIGURE 10. Single channel conductance Λ of protein F from *P. aeruginosa* given as a function of the specific conductance σ of the corresponding aqueous salt solution. The broken line reflects the relation between Λ and specific conductance for *E. coli* and *S. typhimurium* porins.

conductivity as the external solution and assuming a cylindrical pore with a length of 6 nm,[200,201] the average pore diameter d (= 2r) and the cross-section can be calculated according to the equation:

$$\bar{\Lambda} = \sigma \pi r^2/1 \quad (2)$$

Table 3 shows the effective diameter and the cross-section of a number of porin pores from different gram-negative bacteria calculated from the conductance of pores in 1 *M* KCl (σ = 110 msec cm^{-1}). Interestingly, the apparant pore diameters given in Table 3 show excellent agreement with those calculated from the exclusion molecular weight and the liposome swelling assay (see Section E).

It has to be noted, however, that Equation 2 cannot be used to estimate the size of a channel which contains a specific binding site for ions. This can be demonstrated if the properties of the PhoE pore and that of the porin P of *P. aeruginosa* are compared. Figure 11 shows the single channel conductance of both pores as a function of the salt concentration.

Table 3
SINGLE CHANNEL CONDUCTANCE $\bar{\Lambda}$ OF PORIN PORES FROM DIFFERENT GRAM-NEGATIVE BACTERIA IN 1 *M* KCl

Porin	$\bar{\Lambda}$/nS	d/nm	Ref.
	E. coli		
OmpF (B)	2.1	1.2	215
OmpF (K12)	1.9	1.1	215
OmpC	1.5	1.0	215
PhoE	1.8	1.1	126
K	1.5	1.0	215
NmpC	1.3	1.0	215
LamB	2.7	1.4	203
	S. typhimurium		
OmpF	2.2	1.2	70
OmpC	2.4	1.3	70
OmpD	2.5	1.3	70
	P. aeruginosa		
F	5.6	2.0	194
P	0.28	0.6[a]	131
	Y. pestis		
	1.7	1.1	215
	N. gonorrhoeae		
Protein I	—	1.1[b]	190
	A. salmonicida		
	1.6	1.1	214
	A. variabilis		
	3.6	1.6	107

Note: The diameter of the pores is calculated from Equation 2. Assuming a length of the pores of 6NM and a specific conductance of the pore interior of 110 mS cm^{-1}.

[a] Equation 2 cannot be used to calculate the diameter of the protein P channel.
[b] This diameter has been calculated from the single channel conductance in 1 *M* NaCl (1.4 nS).

The PhoE pore shows a linear dependence which is expected for a large water-filled channel with relatively free diffusion of ions within the channel.[126] The porin P channel has a strong 0.6 nm wide selectivity filter for anion.[131,198] This binding site results in saturating single channel conductance with increasing salt concentration. Furthermore, the conductance of the protein P channel is larger than that of the PhoE channel at low salt concentrations although its size is smaller. This may be explained by the existence of a binding site for

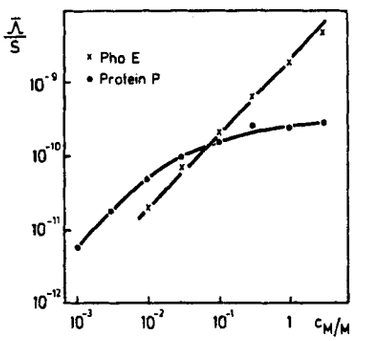


FIGURE 11. Single channel conductance Λ of PhoE porin from *E. coli* and protein P from *P. aeruginosa* as a function of the aqueous KCl concentration. The membranes were formed from diphytanoyl phosphatidylcholine/*n*-decane.

Cl^- with a dissociation constant (kd) of around 30 to 50 m*M*.[126,196] A similar binding site for phosphate inside the PhoE pore as suggested by in vivo experiments seems, on the other hand, to be questionable.[126]

The interaction between the periplasmic binding proteins and the pores in the outer membrane is not fully understood at present. There exists a certain affinity between the maltose binding protein and the maltoporin which has been used to purify the maltoporin.[103,170] An interaction between maltose binding protein and maltoporin is also suggested by experiments on lipid bilayer membranes made from reconstituted vesicles.[202] These experiments indicate that the maltoporin forms only a defined structure in the presence of binding proteins. Furthermore, the interaction of the binding protein with the pore is highly voltage dependent.[202] These results are not easy to understand if they are compared with those obtained from liposomes and lipid bilayers where the maltoporin appears also to form a defined unit in the absence of binding protein.[144,177,203]

3. Ion Selectivity of the Porin Channels

Further information on the structure of the conductance pathway created by the different porins may be obtained by studying the ion selectivity of the pores by zero-current potential measurements. Figure 12 shows the results of such experiments obtained for native and chemically modified OmpF of *E. coli*.[187] The zero-current potential was found to be positive on the more dilute side of the membrane. This indicated that OmpF of *E. coli* forms cation selective pores in lipid bilayer membranes. From the measured V_m and the concentration gradient c″/c′ across the membrane, the ratio P_c to P_a (of the permeabilities P_c for cations and P_a for anions) was calculated according to the Goldman-Hodgkin-Katz equation:

$$V_m = \frac{RT}{F} \ln \frac{P_c c'' + P_a c'}{P_c c' + P_a c''} \quad (3)$$

Table 4 shows the permeability ratios P_c to P_a for the different porins using KCl as the salt in the aqueous phase. The observed slight selectivity for cations or anions may be explained by the presence of negative or positive charges, respectively, in or near the pores. This assumption is supported by the pH-dependence of ion selectivity.[126,199] The permeability ratio P_c to P_a decreases in the presence of a NaCl gradient or KCl gradient if the pH is decreased. Further support arises from selectivity changes of chemically modified porin. OmpF from *E. coli* has completely lost the cation selectivity after amidation (see Figure 12) and PhoE becomes cation selective after acetylation.[204] The chemical modification of the

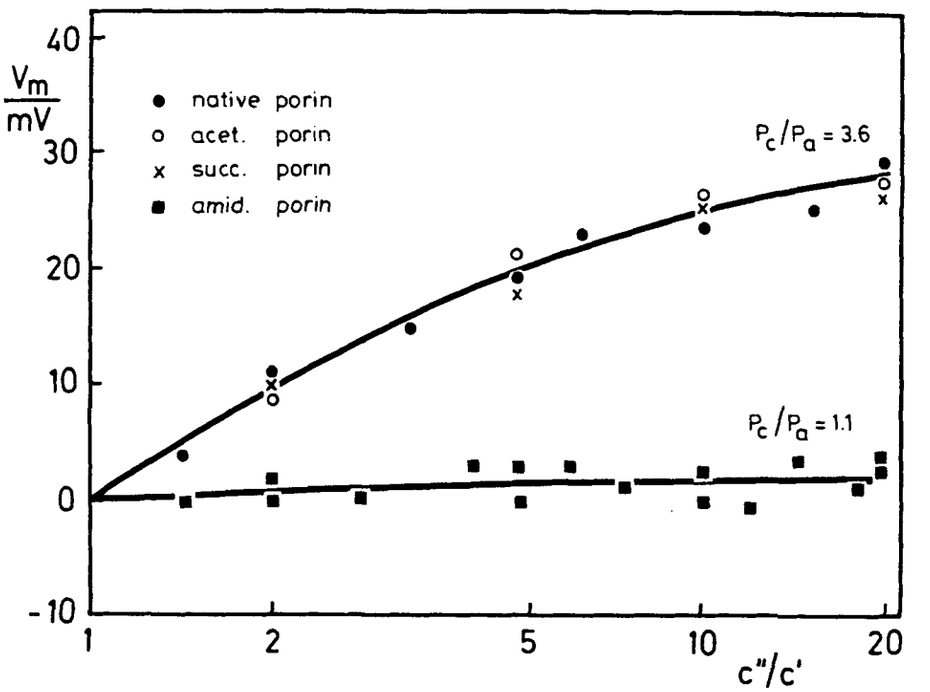


FIGURE 12. Zero-current membrane potentials V_m across membranes from egg-phosphatidylcholine/*n*-decane in the presence of modified and unmodified OmpF from *E. coli* K 12 as a function of the KCl-gradient c"/c' across the membranes. The full lines were drawn according to Equation 3 with the given values of the permeability ratio P_c to P_a.

porins has, on the other hand, only little influence on the single channel conductance of most but not all porin pores. The single channel conductance of large water-filled channels like OmpF and PhoE does not change much after chemical modification,[127,204] whereas it decreases at least ten times for the protein P channel.[198] This finding is another indication that the protein P channel is special among all porin pores of gram-negative bacteria. The experiments with chemically modified porins indicate that the conformation of the trimers remains essentially unchanged by the modification, although physical data on the structure of the modified trimers are not available.

G. Structure of the Porin Pores

Figure 13 shows the primary structures of three porins of *E. coli* outer membrane derived either from the DNA sequence[65-67] or directly from the protein.[64] The amino acid sequences of OmpF, OmpC, and PhoE show an extremely high homology similar to that found for the components of histidine and maltose uptake in *E. coli* and *S. typhimurium*.[206] The homology of the three different porins indicates that they evolved from a common ancestral gene, although the structural genes are located at different positions of the *E. coli* K-12 chromosome (Figure 3). This has also been demonstrated by the construction of a hybrid OmpF-PhoE pore protein.[207] All three porins are acidic proteins which have an excess of 11 negative charges for OmpF, 14 negative charges for OmpC, and 9 negative charges for PhoE. Besides these excess charges, the primary sequence of all three porins contains a large number of negatively and positively charged amino acids. This can be seen from Figure 14 (taken from Reference 208) which shows the distribution of charges along the primary structure of OmpF from *E. coli* B. Most of these charges are presumably not exposed to the surface of the protein or to the pore interior. They form internal ion bridges which

Table 4
ZERO-CURRENT MEMBRANE POTENTIALS V_m FOR DIFFERENT PORINS IN THE PRESENCE OF A TENFOLD KCl GRADIENT

Porin	V_m/mV	P_c/P_a	Ref.
	E. coli		
OmpF (B)	27	3.9	215
OmpF (K12)	26	3.6	187
OmpC	50	26	215
PhoE	−24	0.30	126
K	46	7.6	215
NmpC	−26	0.27	215
	S. typhimurium		
OmpF	46	14	215
OmpC	54	41	215
OmpD	50	23	215
	P. aeruginosa		
F	20	2.7	194
P	−59	<0.01	131
	N. gonorrhoeae		
Protein I	−28	0.28	190
	A. variabilis		
	12	1.7	107

Note: V_m is the electrical potential of the dilute side (10^{-2} *M*) minus the potential on the concentrated side (10^{-1} *M*). The ratios of the permeability P_c (cation) and P_a (anion) were calculated from Equation 3.

stabilize the structure of the pore forming trimer. Intense chemical modification tends in agreement with this to dissociate the trimers into monomers.[186]

Some of the charges are exposed to the surface of the protein or inside the pore and make the pore selective for ions. Their number, however, should be much smaller than has been derived from the chemical modification studies with OmpF porin.[186,209] Lipid bilayer experiments with native and modified OmpF suggest that trimers of this protein do not have positively charged groups exposed to the surface or inside the pore at neutral pH.[187] Otherwise, the negligable influence of acetylation, and succinylation on the pore selectivity cannot be explained (compare Figure 12). The PhoE pore changes its selectivity in the opposite direction after acetylation.[204] This indicates that negatively and positively charged groups are located inside or near the pore. The excess of positively charged groups is responsible for anion selectivity. In fact, the primary sequence of the PhoE protein shows some additional lysines which may be responsible for the change in the pore selectivity from OmpF and OmpC to PhoE.[66,67] Further hybridization experiments between the PhoE and OmpC genes may allow to localize these lysines.[210]

The protein sequence of the maltoporin is also known at present.[138] This protein does not

show a general homology to the other pore proteins in the outer membrane of *E. coli*. Intense comparison of the primary sequence, on the other hand, has suggested some local analogy between major outer membrane proteins which could have to do with their processing and assembly.[95] The pore proteins of *Salmonella* outer membrane show immunological cross-reactions to those of *E. coli*.[119] Although the primary structure of the *Salmonella* porins is not yet known, it can be assumed that there exists a general homology to *E. coli* porins.

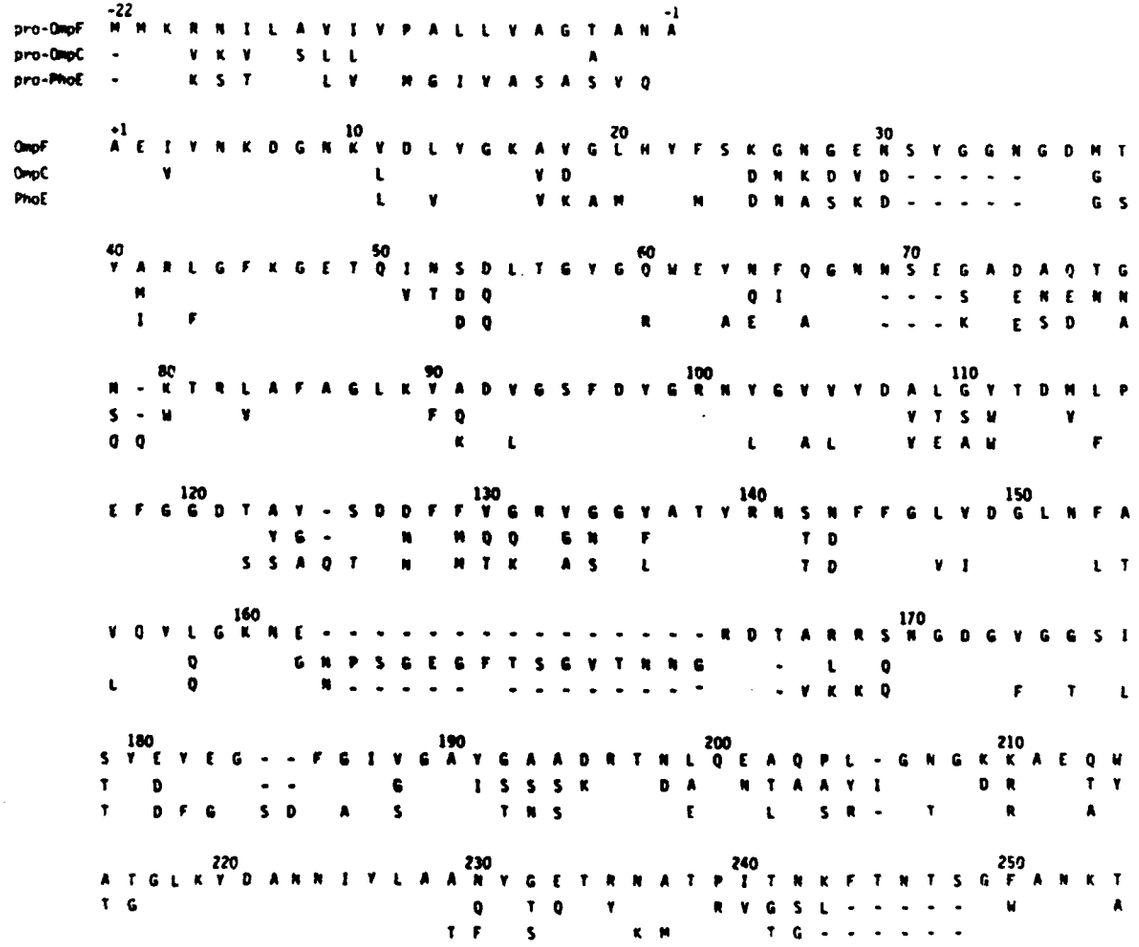
The discussion if the *E. coli* porin trimers contain one or three pores has been controversial in the past. Our own lipid bilayer experiments were always consistent with one voltage-independent pore in a trimer,[196] whereas the results of others have suggested that three independent pores exist in a trimer which show a high cooperativity in the voltage-dependent gating of the single unit.[191,192] It has to be noted, however, that voltage control of outer membrane permeability has not been reported in the literature. The Dounan-equilibrium potential across the outer membrane[21] is by far too low to reach the potentials needed for the gating of the pores. Furthermore, the structure of the OmpF pore as derived from electron microscopical analysis of OmpF embedded in lipid vesicles[201] does not agree with the proposed closing of the pores in a trimer in three identical steps (see below).

The results of the electron microscopic studies suggested that the pore in an OmpF trimer is basically one channel with three openings facing to the outside of the cell.[201] The three channels from the outside merge approximately in the center of the membrane into one channel.[212] This arrangement is shown in Figure 15 taken from Reference 213. Figure 15a shows the OmpF channel from outside with the three openings (which are in agreement with earlier published data), whereas the cross-section given in Figure 15b indicates that these three channels merge into one opening faced towards the periplasmic space. Similar structural studies have not yet been published with the other porins and the maltoporin of *E. coli* K-12. However, because of the close analogy in the primary structure of OmpF, OmpC, and PhoE a very similar structure of the transmembrane channel may be expected for all three porins. Similar considerations apply to the possible structural analogy between *E. coli* and *S. typhimurium* porin trimers.

The OmpF trimer contains only one channel with three openings facing to the outside and a central constriction measuring about 1 nm.[201] The central constriction should be rate limiting for both, the movement of ions and the diffusion of uncharged substrates. The voltage-dependent closing of one of the openings would therefore reduce the current through the pore by only about 5% (and not by 33%). The closing of a second opening would lead to the reduction of the current by another 10 to 20%. This would lead to a rather inhomogeneous pore distribution, which has not been observed in experiments with the OmpF pore. Therefore, it is rather unlikely that the three openings of the OmpF-pore are voltage gated. It seems, moreover, that the whole OmpF trimer is switched off by the action of the high electronic field inside the membrane.[191]

The calculation of an effective diameter from the liposome swelling assay or the single channel conductance data which assumes a cylindrical shape for the pore does not contradict the above-described structure of the OmpF-pore. The diffusion of molecules through any pore structure can in principle be described as if the molecules diffuse through a cylinder with a specific effective diameter. This is supported by the excellent agreement between the effective diameters calculated from the liposome swelling assay and the single channel conductance. This agreement demonstrates also that no basic difference exists between the diffusion of ions and the diffusion of sugars and amino acids through most porin pores.

OmpF trimers have been crystallized using a polyethylene glycol or salt-generated two-phase system.[200] The structure of the OmpF crystals have been analyzed by X-ray diffraction. The maximal dimensions of the trimer are 8 nm × 5.5 nm, which agrees with earlier studies using outer membrane sheets.[211] Furthermore, X-ray diffraction analyses have been found to be consistent with the assumption of β-pleated sheets oriented perpendicular to the membrane plane.[200] This result is in agreement with circular dichroism measurements on *E. coli*[98]



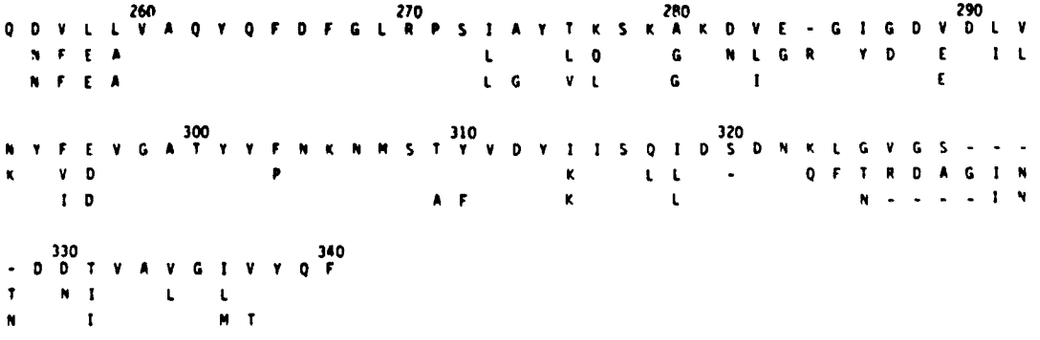


FIGURE 13. Primary structure of three porins of *E. coli* outer membrane. The one-letter code according to IUPAC-IUB Commission on Biochemical Nomenclature was used. (From IUPAC-IUB Commission on Biochemical Nomenclature, *Eur. J. Biochem.*, 5, 151, 1968. With permission.)

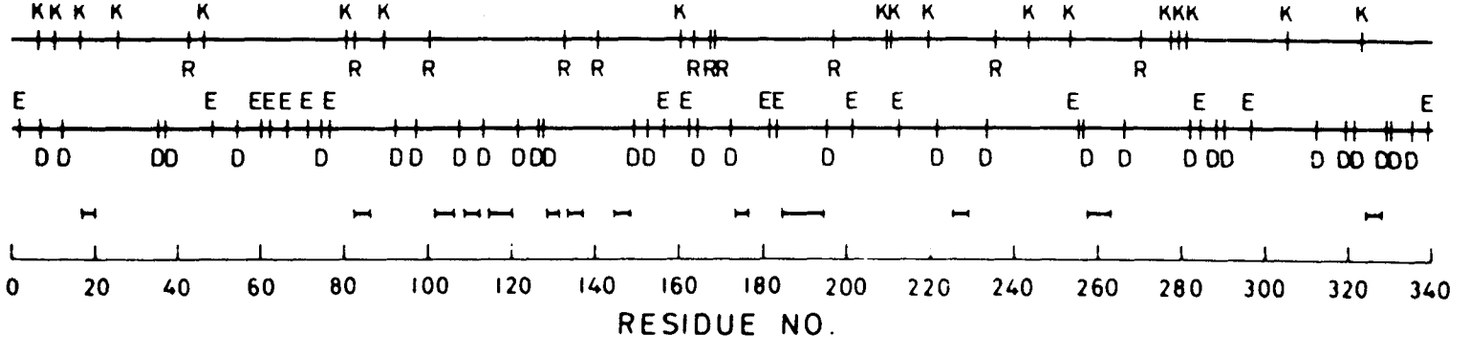


FIGURE 14. Distribution of positively and negatively charged amino acids along the primary structure of OmpF from *E. coli B*. Bottom line: hydrophobic sequences equal to or exceeding four residues (the nonpolar residues alanine and glycine are included). (From Chen, R., Krämer, C., Schmidmayer, W., and Henning, U., *Proc. Natl. Acad. Sci. U.S.A.*, 76, 5014, 1979. With permission.)

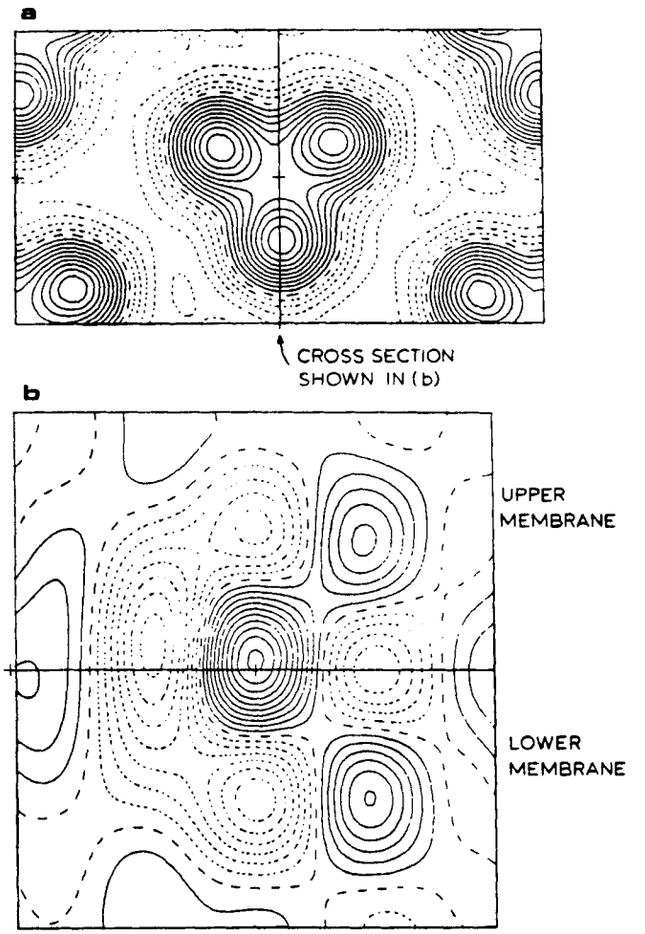


FIGURE 15. (a) Threefold distribution of negative stain, observed as a repeating unit in all three planar crystalline polymorphs of OmpF from *E. coli* B. (b) Section through the apposed presumed pore channels in a double membrane containing regularly packed porin trimers stacked head to head. The transmembrane distribution of dense stain is apparent. (From Dorset, D. L., Engel, A., Massalski, A., and Rosenbusch, J. P., *Biophys. J.*, 45, 128, 1984. By copyright permission of the Biophysical Society.)

and *S. typhimurium*[102] porins where a large fraction of β-sheet structure also has been detected.

The finding that the pore in an OmpF trimer is basically one channel explains why porin monomers are inactive in reconstitution experiments. On the other hand, the existence of three independent channels in a porin trimer in other gram-negative organisms cannot be excluded in all cases. In fact, evidence has been presented that porin trimers in *Rhodopseudomonas spharoides*[106] and *Anabaena variabilis*[107] contain three pores. Porin monomers of these organisms show also membrane activity in the liposome swelling assay and in lipid bilayer membranes.[106,107] The molecular weight of these porins (45,000 to 50,000)[34,106] is considerably larger than that of the porins from enteric bacteria.

The *E. coli* porins show a strong affinity for LPS[200] and it is not clear if LPS is needed for pore-forming activity in reconstituted systems. Porins have been found to be active in the liposome swelling assay without the addition of LPS.[103] But it is not clear if the porins

were completely LPS-free in this case. In another case it has been claimed that the porin is LPS-free, but a rather insensitive sugar test has been used.[169] Folded lipid bilayers from reconstituted vesicles have suggested that LPS is essential for pore formation in the presence of OmpF.[192] On the other hand, completely LPS-free protein I of *N. gonorrhoeae* shows also pore-forming activity.[190]

III. PORINS IN THE OUTER MEMBRANE OF MITOCHONDRIA AND OF THE CHLOROPLAST ENVELOPE

A. Are Mitochondria and Plastids the Descendents of Gram-Negative Bacteria?

Eukaryotic cells have a central organelle, the nucleus. The membrane-enclosed nucleus contains the genetic material of the cell organized into chromosomes. All eukaryotic cells contain, besides the nucleus, mitochondria. Green eukaryotic cells contain plastids in addition where the photosynthesis is located. The aerobic respiration which results in the production of adenosine triphosphate takes place in mitochondria. Both cell organelles contain genetic material which is, as in prokaryotic cells, dispersed throughout the cytoplasm. That means that protein synthesis does not only take place in the cell at the endoplasmic reticulum. Protein synthesis is also located in the cell organelles. However, the DNA and the genetic code of the eukaryotic cell show some differences to those of their organelles. The DNA of mitochondria and plastids has a circular structure similar to that in bacteria.[217,218] Furthermore, the ribosomes of the cell and their organelles are different. Whereas the cell contains ribosomes of a sedimentation constant of 80 S[219] (Svedberg), the ribosomes of mitochondria and plastids are smaller and have a sedimentation constant around 70 S.[220,221] Some antibiotics bind to the 70 S ribosomes of bacteria and inhibit the protein biosynthesis.[222] The same antibiotics also block the protein biosynthesis in mitochondria[223] and plastids[224] but not in eukaryotic cells.[222]

Two different models have been proposed for the origin and the evolution of the eukaryotic cell, the endosymbiotic theory[225,226] and the episome theory.[227] The episome model has in principle only been postulated for the evolution of mitochondria, although it may also be applicable for the evolution of the plastids. This model assumes an advanced aerobic protoeukaryotic cell. The respiration chain was first located in defined sections of its cytoplasmic membrane. These sections invaginated and finally formed topologically closed respiratory organelles by blebbing.[227] A plasmid containing some genes was enclosed and the respiratory organelle was finally surrounded by an outer membrane.

The endosymbiotic model is based on the assumption that the symbiosis of a protoeukaryote with one to two gram-negative prokaryotes led to the first eukaryotic cell. Mitochondria and the plastid envelope have in agreement with this hypothesis an outer membrane, a circular DNA, and ribosomes and they divide in a similar way as bacteria. The first eukaryotic cell may have evolved as early as 1.2 billion years ago.[226,228] This date is based on the increase of the cell size in microfossils and on a time-table estimation of the cytochrome *c* protein sequence data. Cyanobacteria, the proposed ancestors of the plastids, have evolved much earlier because stromatolitic fossiles which indicate the presence of cyanobacteria about 3 billion years ago.[229] Other prokaryotes have presumably evolved much earlier.

There can be only little doubt that the plastids have evolved from an ancestral cyanobacteria as proposed by the endosymbiotic theory. The photosynthetic instrumentation of the chloroplasts[230] and the cyanobacteria is very similar and may have evolved only once. Furthermore, there exists a close analogy between several proteins like ferredoxin, cytochrome *c*, and plastocyanin, and the 5 S ribosomal RNA present in chloroplasts and cyanobacteria (for a review see Reference 226). It has to be noted, however, that the chloroplast DNA encodes only for part of the proteins present in the chloroplast. Recently, it has been shown that some envelope proteins are coded by nuclear genes and that they are synthesized as precursors on soluble cytosolic ribosomes.[231] Since the beginning of the symbiosis more

than one billion years ago some of the plastid genes may have been transferred to the nucleus and new genes have presumably evolved.

The assumption of the endosymbiotic evolution of the mitochondria gives a simple explanation for the existence of an outer membrane, although the function of the outer membrane of gram-negative bacteria and of mitochondria may be quite different. All proteins of the outer membrane of mitochondria are synthesized by the cell and not by the mitochondria.[232] The same is valid for many other proteins located elsewhere like apo-cytochrome *c*, the precursor of cytochrome *c*.[233] This means that, as in the case of the chloroplasts (if the endosymbiotic hypothesis is correct), since the beginning of the symbiosis many genes were transferred to the nucleus and new genes evolved. The rate of the transfer of genes from mitochondria to the nucleus is different for yeast and human eukaryotic cells. That means that certain proteins are encoded in the nucleus of the yeast cell, whereas the genes for the same proteins are located in human mitochondria.[234]

The analysis of the protein sequences of the c-type cytochromes has suggested that the protomitochondria was closely related to the family of gram-negative photosynthetic bacteria, the Rhodospirillaceae.[226] However, earlier[225] and more recent[235,236] investigations of the electron transport chain of the gram-negative bacterium *Paracoccus denitrificans* have indicated that this bacterium and the mitochondria have common ancestors. In fact, partial sequence analysis of the aa_3 cytochrome oxidase of *P. dentrificans* shows a striking homology to the mitochondria encoded subunits I and II of eukaryotic cells.[236] Although the cytochrome oxidase of this bacterium has only two subunits as compared with the twelve polypeptide chains of mammalian types,[237] it shows the same function and it reacts with mammalian-type cytochrome *c*.[235] This clearly indicates together with the analysis of the 5 S ribosomal RNA of the eukaryotic host that the endosymbiotic theory is also very likely for the evolution of mitochondria.[226]

B. Structure and Composition of the Outer Mitochondrial Membrane

The matrix of mitochondria is surrounded by two different membranes, the inner and the outer membrane. The inner membrane shows invaginations (the cristae) and its surface is much larger than that of the outer membrane. The electron transport chain, located in the inner membrane, pumps protons in the intracristal space (which is *in situ* very small; see below) during aerobic respiration. The electrochemical gradient of protons is used by the F_o F_1 ATPase for the synthesis of ATP (in the matrix space). The ATP is transported via the ATP-ADP antiporter into the intracristal space and leaves the mitochondria through the outer membrane. The substrates of the respiration chain (mostly anionic) permeate also through the outer membrane. Enzymes like the adenyl kinase are also located in the peripheral space between inner and outer membrane.

Mitochondria show *in situ* a different structure than after isolation.[238] There does not exist the conventional intercristal space or peripheral space in mitochondria from rapidly frozen tissue. Inner and outer membrane appear in this case as a five-layered 120-Å-thick structure with close contacts similar to tight junctions between inner and outer membrane.[239] The cristal membranes are also closely apposed. Isolated mitochondria have large intercristal and peripheral spaces, although most close contacts between inner and outer membranes are retained.[36] Recently, it has been shown that the number of contact sites between inner and outer membrane are dependent on the energetization of the mitochondria.[37,240] These contacts are presumably responsible for the substantial loss of outer membrane during isolation and for the contamination of outer membrane fractions with inner membrane.[241]

1. Isolation of Outer Mitochondrial Membrane

Many different methods have been described for the isolation of mitochondria from rat liver, yeast, *N. crassa*, and other sources.[241-243] Here only the isolation and purification of outer membrane from rat liver mitochondria will be described in some detail. The method

is based on the osmotic procedure proposed by Sottocasa and co-workers.[244,245] Mitochondria are suspended in a buffer with a low osmolarity (20 mosmol). Subsequently, sucrose is added to the suspension in a final concentration of 0.9 *M*. Because sucrose is permeable through the outer membrane but not through the inner membrane, the matrix shrinks. The mitochondria are then sonicated for a short time. The sonication procedure has been found to be very critical for the purity of the outer membrane preparation. The ruptured mitochondria are diluted and layered on top of a step sucrose gradient with 40 and 26% sucrose. After centrifugation for several hours the outer membranes are obtained as a band in the gradient, whereas the pellet contains the inner membrane. A purer outer membrane may be obtained by a second sucrose step gradient centrifugation.[245] The contamination of the outer membrane with endoplasmic reticulum, peroxisomes, lysosomes, and inner membrane can be calculated from the activities of the corresponding marker enzymes as compared with the marker enzymes of the outer membrane (monoamine oxidase, antimycin A-insensitive NADH-cytochrome *c* reductase, and kynurenine 3-hydroxylase).

2. Lipid Composition

The lipid compositions of outer and inner mitochondrial membranes are different. This means that the close contacts of outer and inner membrane do not allow a rapid exchange of lipids. The major phospholipid of the outer membrane is phosphatidylcholine which is at present between 50 and 60% of the total phospholipid content, whereas the inner membrane contains about 40% of this phospholipid.[246,247] Phosphatidylethanolamine is present in the outer membrane at a level of about 20 to 30%.[246-248] Besides these two neutral phospholipids, the outer membrane also contains some negatively charged lipids like phosphatidylinositol and phosphatidylserine in a total amount of 10 to 20%.[247,248] Cardiolipin is presumably not present in the outer membrane although it is a major lipid of the inner membrane (about 20%).[248] On the other hand, the inner membrane of rat liver mitochondria does not contain cholesterol, whereas this lipid is present in the outer membrane in a molar ratio of cholesterol to phospholipid of 1:9.[248] The outer mitochondrial membrane of other organisms like plants may contain other sterols. Ergosterol has been found in a high concentration in *Neurospora* mitochondria.[249]

3. Proteins

A large number of enzyme activities have been found to be located in mitochondria. Some of the enzymes are present in the outer membrane and are used as marker enzymes. The monoamine oxidase is one enzyme found preferentially in mammalian tissue,[250] whereas the kynurenine hydroxylase also has been identified in outer mitochondrial membrane of yeast[251] and *Neurospora*.[252] Occasionally, the antimycin insensitive NADH-cytochrome *c* reductase has also been used as a marker enzyme.[245] The polypeptide pattern of the outer membrane from yeast mitochondria has been studied in a recent publication.[251] Ten different polypeptides were identified with four prominent bands of mol wt 14,000, 29,000, 45,000, and 70,000. The protein pattern was found to be typical for this membrane. Microsomes and inner membranes did not contain any of the prominent proteins of the outer membrane. Only the function of one of these proteins is known. The mol wt 29,000 polypeptide is the general diffusion pathway in the outer membrane of yeast mitochondria.[253,254] The general diffusion pathways in the outer mitochondrial membrane of other organisms have molecular weights between 30,000 and 35,000.[254-259] The mitochondrial porin represents the only protein in the membrane with known functions as a general diffusion pore[258,260] and as a binding protein for hexokinase[261,262] and glycerokinase.[263]

C. Function of the Outer Mitochondrial Membrane

The outer mitochondrial membrane contains a number of enzymes which are involved in the metabolism of amino acids and fatty acids. These enzyme activities do not presumably

represent the major function of the membrane. The outer membrane acts as a permeability barrier for hydrophilic solutes with a defined exclusion limit.[254-260] As this pathway is voltage-dependent, the exclusion limit could be regulated which could in turn regulate the mitochondria. However, no evidence has been found so far for such a regulation process. In fact, the pore diameter appears too large and the pore selectivity is too small for an ionic gradient to be stable for a longer time.[255,256] A change in the membrane asymmetry of the outer membrane would be too slow to account for a rapid regulation process. But we have to keep in mind that the in vitro mitochondria have a different appearance than the *in situ* mitochondria, where all membranes are closely apposed.[239] This close apposition could result in an electric coupling between inner and outer membrane *in situ*, which could lead to a change in the permeability of the outer membrane for substrates of the oxidative phosphorylation and its products. This possibility is supported by the finding that *all* mitochondrial pores are voltage dependent in a similar fashion although their origin and primary sequence may be different.

Another major function of the outer mitochondrial membrane is most probably the compartmentation.[240] It has been observed that the hexokinase bound to mitochondrial surface has a higher activity with ATP coming from the oxidative phosphorylation as compared with cytoplasmic ATP.[264,265] The hexokinase uses the ATP from oxidative phosphorylation directly and does not allow a equalibration with the extramitochondrial pool. This finding is understood at present because the hexokinase (and glycerokinase) binding protein is identical with the pore.[261,263,266] Furthermore, a microcompartmentation between hexokinase and adenylate translocator has been observed in energized mitochondria, which excludes the adenylate kinase from the ATP-pool in energized mitochondria.[240] A similar microcompartmentation has also been proposed for glycerolphosphate and acyl-CoA.[240]

D. Biosynthesis of Mitochondrial Porin

The DNA of mitochondria encodes only for a small part of total mitochondrial protein. The genes of the other proteins are located in the nucleus of the cell and their biosynthesis takes place outside the mitochondria on cytoplasmic ribosomes. Different pathways have been observed for their import into the matrix space, the inner membrane, the intermembrane space and the outer membrane (see Reference 232 for a recent review). The biosynthesis and assembly of the mitochondrial porin of *Neurospora crassa*[267] and the yeast *Saccharomyces cerevisiae*[253,268] have been studied in detail. The precursor of the mitochondrial porin is synthesized on cytoplasmic ribosomes with the same molecular weight as the mature protein.[253,267,268] This means that no NH_2 cleavage of an amino terminal leader sequence occurs during or after the insertion process into the outer membrane. Studies with in vitro synthesized proporin and isolated mitochondria suggest a binding of this protein to the surface of the outer mitochondrial membrane.[267]

The receptor for the mitochondrial porin on the surface of mitochondria has not been identified and isolated until now. Nevertheless, little doubt exists that such a receptor must exist on the outer membrane. First, the binding of proporin to the surface of mitochondria has been found to be proteinase sensitive[267] (although not in all cases[268]) and, second, the unspecific binding and the insertion of mitochondrial porin into the cytoplasmic membrane would be a disaster for the cell. Porin has, in agreement with this, only been found to be located in the outer mitochondrial membrane.[269] Proporin from *N. crassa* shows insertion into rat liver and yeast mitochondria but it does not insert into plasma membranes of human fibroblasts.[267] Energization of mitochondria is not required for this process. The pore is presumably formed from two to three proporin molecules, sterols, and lipids. The mature porin in this complex is no longer sensitive to proteinases as is the bound proporin.[253,267,268]

E. Isolation and Purification of Mitochondrial Porin

Mitochondrial porins have been isolated from a number of different mitochondria like rat

liver,[256.257.259] pig heart,[270] *N. crassa*,[255.271.272] the yeast *Saccharomyces cerevisiae*,[253] and mung bean.[258] The first step in some isolation procedures was the isolation of outer membrane (Section III.B.1). Other procedures used whole mitochondria. The latter method avoids the substantial loss of mitochondrial porin during the isolation procedure of the outer membrane.[243] The explanations for the loss are presumably an incomplete separation between both membranes because of the tight local association between inner and outer membrane.[36.37] In the following paragraphs, several different methods for the isolation of mitochondrial porin will be described.

Outer membrane from rat liver shows on SDS gel electrophoresis four "major" bands and a large number of "minor" bands.[256.257] The outer membranes are first washed with 2% Triton® X-100 solution. The residual pellet is then solubilized in an aqueous solution of 2% Triton® X-100 and 1*M* NaCl. The crude porin is further purified by chromatography across a DEAE-Sepharose column followed by chromatography across a CM-Sepharose column.[257] The protein has been found to be active in the vesicle permeability assay similar to that published earlier for *S. typhimurium* porins (see Section II.E.1). The [^{14}C] dextran to [^{3}H] sucrose ratio was largest for the protein obtained from the CM-Sepharose column, although there did not exist a linear relationship between the retained [^{3}H] sucrose and the protein concentration.[257] This is easy to understand because one pore per vesicle is sufficient for the release of all [^{3}H] sucrose.

Another method for the purification of mitochondrial porin from rat liver starts from whole mitochondria, which are solubilized by adding Triton® X-100 to a final concentration of 2% (v/v).[259] The supernatant of a centrifugation step is applied to a mixed DEAE-Sephadex and CM-Sephadex 1:1 (w/w) column. The eluate is subsequently applied to a Sepharose CL-6B column and finally to an affinity column which contains as active material concanavalin A bound to Sepharose 4B. The column is washed and finally eluted with 0.3 *M* α-mannoside. The activity of the fractions can be tested using the lipid bilayer assay.[259] The protein purification obtained using the ConA-column remains obscure so far because neither the mitochondrial porin from rat liver[257] nor that from *N. crassa*[255] has been reported to be a glycoprotein. The same consideration apply to the inhibition of the membrane activity of mitochondrial porin by concanavalin A (ConA).[273]

Based on lipid bilayer experiments,[261] two-dimensional electrophoresis,[262] and peptide mapping,[262] the mitochondrial porin has been found to be identical with the hexokinase binding protein located in the outer membrane of rat liver mitochondria.[261.262] Thus, the purification method of the hexokinase binding protein, as has been published by Felgner et al.,[266] can also be used for the isolation of the mitochondrial porin. This method is based on solubilization of outer membrane in octylglycoside (final concentration 2.6%). The supernatant of a subsequent centrifugation step is dialyzed against a buffer. Vesicles form which show hexokinase binding and which are enriched in a protein of mol wt 30,000. Then the procedure is repeated (2 × reconstituted) and the vesicles are subjected to sucrose density gradient centrifugation. The centrifugation resulted in a homogeneous preparation of the hexokinase binding protein[266] (and the mitochondrial porin[261]).

The outer mitochondrial membranes of *S. cerevisiae* and *N. crassa* contain a smaller number of proteins than that of mammalian tissue. The dominant band is the mitochondrial porin of mol wt 30,000 which comprises about 20% of total outer membrane protein. Thus, the isolation procedure of the mitochondrial porin from both organisms is relatively simple.[255.272] Outer mitochondrial membranes from *N. crassa* are washed in buffer solution containing 30 m*M* octylglycoside, which removes most of the minor proteins. The pellet is extracted with Genapol® X-100 (a detergent which is very similar to Triton® X-100 but which contains isotridecyl groups instead of aromatic rings) and the supernatant is applied to a DEAE-cellulose column as a final purification step.[255.272]

The inner mitochondrial membrane is not solubilized by the detergent Genapol® X-100.[274] Thus, this detergent can be used for rapid isolation and purification of mitochondrial porin

from whole mitochondria.[271,272] The mitochondria are extracted with a buffer containing 2.5% Genapol® X-100. The supernatant of the subsequent centrifugation is applied to a dry hydroxyapatite (HTP) column. The eluate is then passed through a dry column filled with hydroxyapatite and celite (1:1 w/w). The eluate of this column contains porin with minor contaminants which can be removed by chromatography across DEAE-cellulose.[271,272] This method is generally applicable and results in excellent porin preparations for mitochondria from yeast[254] and from *Paramecium*.[275] Recently, the dry HTP column followed by chromatography across organomercurial agarose and across mixed dry HTP/celite has been used to purify the 35-k dicyclohexylcarbodiimide (DCCD) reactive protein from beef heart mitochondria.[270] This protein has been identified to be identical with the mitochondrial porin on the basis of the membrane activity in lipid bilayer experiments.[276] This protein is most probably located in the outer mitochondrial membrane and it is not identical to the phosphate carrier or the F_o component of the H^+-ATPase.[276]

It seems that HTP column chromatography results in porin of high purity in the case of whole mitochondria or outer mitochondrial membranes from yeast,[254] *Paramecium*,[275] and *Neurospora*,[271,272] presumably because of the limited number of prominent bands and of the high content of porin. Mitochondrial porin of high purity from mammalian tissue is obtained by chromatography across DEAE Sepharose and across CM-Sepharose.[257] The procedure should start in this case from outer membranes and not from whole mitochondria.

F. Reconstitution of Mitochondrial Porin

1. Reconstitution into Lipid Vesicles

Mitochondrial porins from mung bean,[258] rat liver,[257] and *Neurospora crassa*[277] have been reconstituted into liposomes and lipid vesicles. The reconstitution of mitochondrial porin from mung bean into liposomes was essentially the same as described in Section II.E.1 for the reconstitution of bacterial porin.[99,103] The porin containing liposomes were loaded with two radioactively labeled solutes, [^{14}C] sucrose and [^{3}H] dextran. The [^{14}C] sucrose has been found to permeate the membranes, whereas the dextran was retained indicating an enclosed volume for the liposomes. The exclusion limit of the pore was measured using a variety of different radioactively labeled solutes. But in contrast to measurements with bacterial porins,[99] the permeability of the liposomes was measured in the absence of dextran. The exclusion limit was not very sharp and the "cut-off" curve was smeared out between mol wt 1000 and 10,000.[258] It has been suggested that the curve is smeared out because of impurities in the solutes and that the true cut-off limit lies between mol wt 4000 and 6000.[258]

The exclusion limit of mitochondrial porin from *Neurospora crassa* has been measured using a liposome swelling assay.[277] The liposomes were prepared from phospholipid and a crude protein fraction from mitochondria. The liposomes have been found to respond to the osmolarity of the environment. When transferred into a hypertonic sucrose solution, the liposomes showed first shrinkage and then swelling because sucrose is permeable through the porin pores. This procedure was repeated for a number of sugars and polyethylene glycols of different molecular weights. Polyethylene glycols with molecular weights of about 1400 and of about 3400 have been found to be permeable through the mitochondrial pore, because the shrinkage was always followed by reswelling, whereas no reswelling was observed in the presence of polyethylene glycol of mol wt 6800.[277] Using this result, the diameter of the mitochondrial pore has been calculated to be 4 nm. It has to be noted that this osmotic method is, by far, not as accurate as the swelling method used for the bacterial porins[144] (see Section II.E.2). In fact, structural studies[278] and lipid bilayer experiments[255,256] suggest that the diameter of the mitochondrial pore is much smaller and that it has only a value of about 2 nm.

2. Reconstitution into Artificial Lipid Bilayer Membranes

Mitochondrial porin from different sources has been reconstituted into artificial lipid

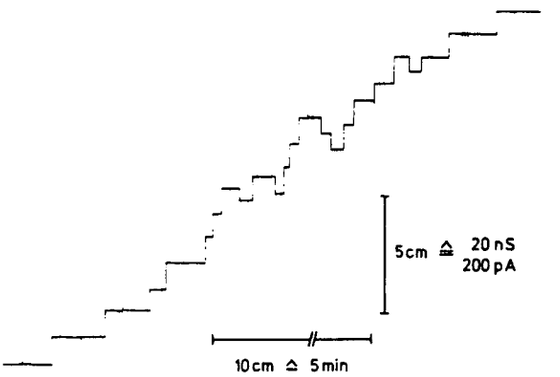


FIGURE 16. Step-wise increase of the membrane current in the presence of 20 ng/mℓ mitochondrial porin from yeast added to the aqueous phase containing 1 *M* KCl, pH 6. The membrane was formed from diphytanoyl phosphatidylcholine/*n*-decane; V_m = 10 mV.

bilayer membranes. Schein et al.[279] were the first to notice that a channel-forming activity was present in mitochondria but not in other membrane fractions of *Paramecium aurelia*. Because of the voltage dependence and the anion selectivity of the channel they used the name "voltage-dependent anion-selective channel" (VDAC). Schein et al.[279] have suggested that the VDAC is located in the inner membrane and that it has to do with the high chloride permeability observed in mitochondria from brown adipose tissue.[280] However, Colombini[260] showed later that the VDAC was located in the outer mitochondrial membrane.

Here the name mitochondrial porin is used instead of VDAC. The mitochondrial porin forms general diffusion pores with a very limited selectivity for anions (see below). Furthermore, gram-negative bacteria are presumably the ancestors of mitochondria and the name porin has widely been accepted for the general diffusion pathway in their outer membranes and in those of mitochondria.[255-258]

The properties of mitochondrial porins in lipid bilayer membranes have been studied by adding the protein to the aqueous phase bathing a membrane made of different lipids. Colombini and co-workers[259,260,279] used always solvent-free lipid bilayer membranes in their reconstitution experiments, whereas all other studies were performed with solvent-containing lipid bilayer membranes.[254-256,275,276] It has to be noted, however, that the results of both types of experimental conditions show, in contrast to the experiments with bacterial porins, good agreement. The addition of small quantities of mitochondrial porin to preformed lipid bilayer membranes results in a strong time-dependent increase of the membrane conductance.[255,256,259,260] The channel-forming activity has either been characterized as the formation of channels per unit time and per protein weight[277] or as the specific membrane conductance a certain time after addition of a fixed protein concentration.

3. Single Channel Conductance

The addition of much smaller quantities to membranes of small areas results in a step-wise increase of the membrane conductance (Figure 16). Mitochondrial porin from yeast was added in a concentration of 20 ng/mℓ to a black lipid bilayer membrane from diphytanoyl phosphatidylcholine/*n*-decane. Most current steps were directed upward, and terminating events were only rarely observed at low transmembrane potentials. Histograms showed a rather uniform distribution for the current fluctuations with a typical average single channel conductance for mitochondrial porin from rat liver[256] and *Neurospora crassa*[255,259] of about 4.5 nS (see Table 5). Only a limited number of smaller steps were observed (see Figure

Table 5
SINGLE CHANNEL CONDUCTANCE ($\bar{\Lambda}$) OF MITOCHONDRIAL PORIN AND CHLOROPLAST PORIN PORES FROM DIFFERENT SOURCES IN 1 *M* KCl

Pore	$\bar{\Lambda}$/nS	d/nm	Ref.
Mitochondrial porin			
Neurospora crassa	4.5	1.8	277
	5.0	1.9	255
Rat liver	4.3	1.7	256
	4.2	1.7	259
Pig heart	4.0	1.7	275
Yeast	4.2	1.7	254
Paramecium	2.4	1.3	275
Chloroplast porin			
Spinach	7.0	2.2	18

Note: The diameter of the pore (d) is calculated from Equation 2. Assuming a length of the pores of 6 nm and a specific conductance σ of the pore interior of 110 mS cm^{-1}.

16). These smaller steps have been explained as substates of the pore. However, they are very frequently observed under certain conditions and in lipid bilayer experiments in the presence of porin from *Paramecium*, only the small pores with a single channel conductance around 2.4 nS have been observed (Table 5).[275] This result indicates that the mitochondrial pore has presumably two different stable conformations.

The channel in the outer mitochondrial membrane is permeable for a variety of different ions. Table 6 shows the single channel conductance of rat liver mitochondrial porin in the presence of different salt solutions.[256] Although there is a considerable change in the conductance of the pore, the ratio $\bar{\Lambda}/\sigma$ varied very little. This result allows a rough estimate of the channel size on the basis of Equation 2. Assuming a pore length of 6 nm, the diameter of most pores is about 1.8 nm (Table 5), which would allow the permeation of solutes up to a molecular weight of 2,500.

4. Selectivity of the Mitochondrial Porin

The mitochondrial porins have been identified as basic proteins with isoelectric points between pH 7 and 8.[257,258] The weak anion selectivity of the porin pore is presumably caused by an excess of positively charged groups at the rim or inside the pore. The zero-current membrane potentials are for a tenfold KCl-gradient on the order of -10 mV at the more dilute side,[256,259] which corresponds to a twofold higher permeability for Cl^- over K^+ (Equation 3). Schein et al.[279] have reported a much larger anion selectivity, but this large selectivity has not been confirmed by others. The argument that the difference is caused by the different states of the pore is not convincing because no discontinuity has been observed in zero-current membrane potential measurements. The ionic selectivity of the mitochondrial porin can be inverted by succinylation.[281,282] This indicates that lysines exposed to the surface or inside the pore are responsible for its selectivity.

5. The Mitochondrial Pore is Voltage Dependent

Schein et al.[279] have already found that the mitochondrial porin forms voltage-dependent

Table 6
SINGLE CHANNEL CONDUCTANCE ($\bar{\Lambda}$) OF THE MITOCHONDRIAL PORE FROM RAT LIVER IN DIFFERENT SALT SOLUTIONS

Salt	c/M	$\bar{\Lambda}$/nS	$\bar{\Lambda}/\sigma/10^{-8}$ cm
KCl	0.01	0.05	3.6
	0.03	0.15	3.8
	0.1	0.48	3.7
	0.3	1.3	3.6
	1	4.4	4.0
NaCl	1	3.8	4.5
LiCl	1	3.4	4.8
RbCl	1	4.2	3.5
$MgCl_2$	0.5	2.7	4.2
K_2SO_4	0.5	2.4	3.2
$Tris^+$ $Hepes^-$ (pH8)	0.5	0.23	3.2
$N(C_2H_5)^+$ $Hepes^-$	0.5	0.17	3.5

Note: σ is the specific conductance of the corresponding salt solution.

From Roos, N., Benz, R., and Brdiczka, D., *Biochim. Biophys. Acta*, 686, 204, 1982. With permission.

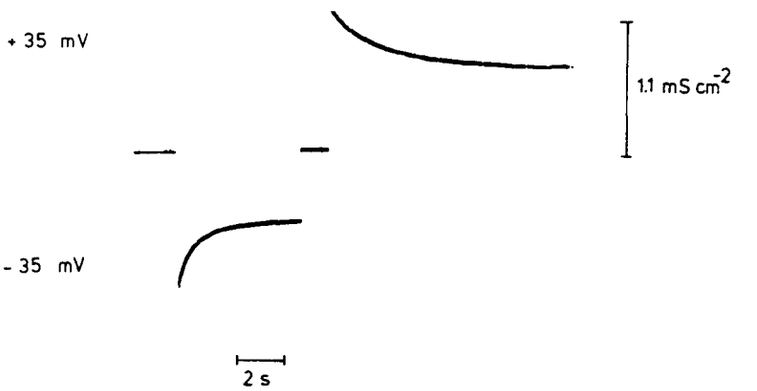


FIGURE 17. Decay of the specific membrane conductance as a result of transmembrane potentials of opposite sign. Mitochondrial porin from yeast was added in a concentration of 500 ng/mℓ to the cis side of a membrane from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 1 *M* KCl.

pores in lipid bilayer membranes. This observation has later been confirmed and all mitochondrial porins investigated so far show a decreasing single channel conductance as a result of increasing voltage.[254-257,275,276] Whereas it has been reported first that the mitochondrial pore has only two states, one open and one closed, it is now more or less obvious that there exist in most cases many different closed states of various amplitudes. Multiple closed states have been found for mitochondrial porins from *N. crassa*,[255,273] yeast,[254] rat liver,[256] and pig heart,[276] but not for *Paramecium*.[275] The existence of multiple closed states and the incomplete closings of the pores are the reasons why in multichannel systems the membrane current does not completely decay to zero. This is shown in Figure 17 where transmembrane potentials of 35 and -35 mV were applied to a membrane containing many mitochondrial pores from yeast ($5 \cdot 10^5$ pores per square centimeter). The membrane conductance decreases

Table 7
AMINO ACID COMPOSITION OF MITOCHONDRIAL PORIN FROM RAT LIVER[257] AND *NEUROSPORA CRASSA*[255]

Amino acid	Porin from rat liver (mol. %)	Porin from *N. crassa* (mol. %)	PhoE from *E. coli* (mol. %)
Lys	9.44	8.55	6.97
His	1.35	2.90	0.30
Arg	2.27	2.37	3.64
Asx (+Asp)	11.61	11.08	17.58
Thr	8.96	9.81	6.97
Ser	5.83	7.71	5.46
Glx (+Glu)	8.29	2.92	9.09
Pro	2.51	2.62	0.91
Gly	11.65	9.39	10.91
Ala	7.38	13.31	8.18
Cys	ND	ND	0
Val	5.93	7.56	3.64
Met	0.59	2.03	2.12
Ile	3.65	3.86	3.94
Leu	9.97	5.93	6.36
Tyr	4.41	3.33	6.67
Phe	6.16	6.64	6.36
Trp	ND	ND	0.91

Note: The amino acid composition of PhoE from *E. coli*[67] is given for comparison.

as a result of the voltage in a somewhat asymmetric fashion. This shift of the midpoint potential of -5 to -10 mV (as referred to the cis side, the side of the addition of the protein) has been observed in many different systems.[254-256] It is presumably the result of the somewhat asymmetric insertion of the mitochondrial porin if it is added to only one side of the membrane. The lower conductance states of the mitochondrial pore have a different permeability and selectivity than the open pore. In the case of *Paramecium* mitochondrial porin, the lower conductance shows a small permeability for $Tris^+$ and a cation selectivity.[275]

Doving and Colombini[281,282] studied the influence of chemical modification on the voltage dependence of the mitochondrial pore. Succinylation of the pore protein led to a voltage-independent pore. This indicates that lysines are presumably responsible for the 3 to 5 charges which have been found to be associated with the gating process.[277,279] From the result of asymmetric addition of succinic anhydrate to both sides of the membrane, it has been concluded that only one gate is responsible for the voltage dependence of the mitochondrial pore.[282] However, a two-gate model similar to that proposed for the voltage dependence of the gap junctions[283] seems to be more consistent with the experimental data.

G. Structure of the Mitochondrial Porin

1. Amino Acid Composition

To date, no mitochondrial porins have been sequenced. Thus, it is not possible to compare the structure of mitochondrial porins with that of the bacterial porins. On the other hand, the apparent molecular weight of the mitochondrial porin is similar to that of the bacterial porins. Similar considerations apply to the amino acid composition of both types of porins. Table 7 shows the amino acid composition of two mitochondrial porins and of PhoE from *E. coli*. The mitochondrial porins are composed of about 46% polar amino acids (according to the definition of Capaldi and Vanderkoi[284]). A similar value has also been found for porins

of *E. coli* [98] and *S. typhimurium*.[69] The low hydrophicity of both types of porin is presumably related to their biosynthesis. Both are probably synthesized as water soluble precursors, and both do not contain carbohydrates. In this respect, it is interesting to note that mitochondrial porin from *Neurospora crassa* becomes water soluble after special treatment and binds in this form to the surface of mitochondria as the precursor does.[285] The water-soluble porin forms pores in lipid bilayer membranes in the presence of sterols and phosphatidylcholine. Their properties have been found to be very similar to those observed for the isolated porin.[285]

Recently, the mitochondrial porin from pig heart has been identified to be identical to the 33-k DCCD binding protein of mitochondria.[276] This means that the pore protein contains a negatively charged group located in a hydrophobic environment. Furthermore, cysteines have been detected in the amino acid analysis of this 33 k-DCCD binding protein. These cysteines react with *N*-ethylmaleimide (NEM), suggesting that they are not involved in sulfhydryl bridges.[276]

2. Arrangement of the Pores in the Outer Mitochondrial Membranes

The pores in the outer mitochondrial membranes have also been studied using morphological approaches like electron microscopy and X-ray diffraction. Negative staining of isolated outer membranes from mung bean mitochondria have revealed stain filled pits of 2- to 3-nm diameter.[286] This finding has been confirmed by studies of the outer membrane of *Neurospora crassa* mitochondria.[278,287] Furthermore, it has been found that the pores are organized in a crystalline structure if the outer membranes are dialyzed against low salt buffer and lose some phospholipids.[278,288,289] The repeating unit contains three pores (see upper part of Figure 18) which can be visualized using Fourier-filtered electron microscopic images.[278] The existence of three pores in a single unit is consistent with the observation that mitochondrial porin from a crude outer membrane fraction inserts in lipid bilayer membranes as triplets,[290] whereas isolated and purified porin inserts in monomers.[255] X-ray diffraction studies on pellets of plant outer mitochondrial membranes suggest that the pore forms a prominent structure with an outer diameter of 5 nm and a low-electron-density pore interior of 1.8 to 2 nm.[291,292] It has to be noted that these pore dimensions are in excellent agreement with the diameter calculated from the single channel conductance, but they disagree with the liposome swelling assay.[273,276] The three-dimensional structure of the pore in the outer mitochondrial membrane of *N. crassa* has recently been resolved from the crystalline arrays embedded in uranyl acetate.[293] Electron microscope image reconstruction show that the mitochondrial pore is a cylinder normal to the membrane plane with a diameter of about 2.5 nm. A similar structure has also been observed in the outer mitochondrial membrane from rat liver.[293] This means that the mitochondrial pore is quite different to the channel in the OmpF trimers from the outer membrane of *E. coli* (compare Section II.G), although it cannot be excluded that bacterial pores are cylinders in certain cases.

So far, it is not clear if the pores in the outer mitochondrial membrane of all eukaryotic cells are organized as trimers which would show some analogy to the bacterial pores. Furthermore, it is still puzzling how many subunits form a pore. Sedimentation experiments would be consistent with a dimer,[294] whereas gel filtration suggested a trimer.[273] Recent cross-linking experiments performed with outer mitochondrial membrane from yeast also provide evidence for dimers, although a small amount of cross-linked trimers have also been observed. On the other hand, porin monomers can also be cross-linked to a protein of mol wt 14,000 of outer mitochondrial membranes, which indicates a close neighborhood of both proteins.[295] The experiments performed with water-soluble mitochondrial porin suggest that lipid and sterol are also required for the formation of the single conductive unit.[285]

H. Exclusive Limit of the Pore in the Outer Membrane of the Chloroplast Envelope

The outer membrane of the chloroplast envelope has been found to be permeable to small solutes but not to dextrans of large molecular weight.[206] This suggested sieving properties

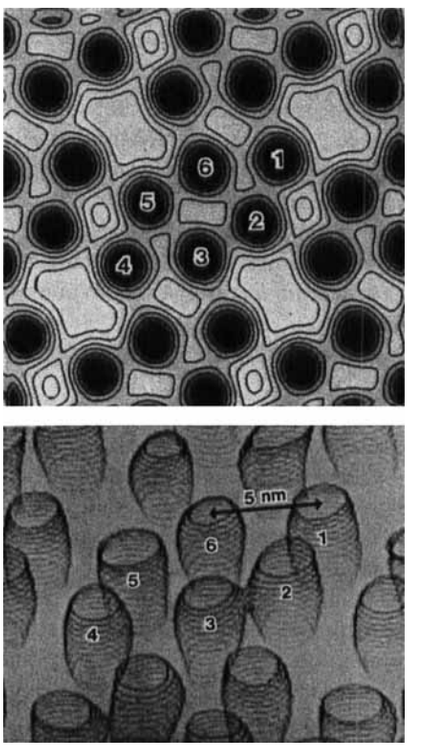


FIGURE 18. Averaged electron microscopic image of potassium phosphotungstate (KPT) stained outer membrane channel arrays of *Neurospora crassa* mitochondria (top). Three-dimensional contour map of the *N. crassa* crystalline array (bottom), showing the boundaries of the stain cylinders. (Courtesy of Dr. Mannella.)

for this membrane. The exact exclusion limit for the penetration of the outer membrane has recently been measured.[18] Plasmolyzed spinach chloroplasts were incubated in radioactive labeled solutes of different molecular weight. Then the chloroplasts were subjected to silicone oil filtering centrifugation and the radioactivity of the lower phase was examined.[18] The results are shown in Figure 19. Polypeptides up to a molecular weight of more than 6500 were permeable through the outer membrane into the intermembrane space. The exclusion limit of the pore lies between molecular weights of about 7000 to 13,000, probably around 9000 to 10,000. This corresponds to a pore diameter of about 3 nm. This large diameter of the chloroplast porin pore is consistent with the result of lipid bilayer membranes. Reconstitution experiments in the presence of purified outer membrane of the chloroplast envelope resulted in an appearance of single pores with an average single channel conductance of about 7 nS in 1 *M* KCl[18] (compare Table 5). This is the largest pore of the porin type (i.e., of general diffusion pores) observed so far in the outer membrane of gram-negative bacteria and of cell organelles.

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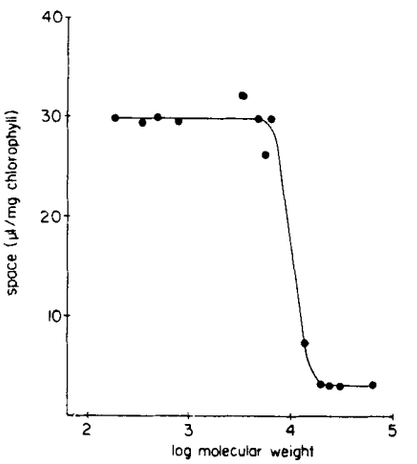

FIGURE 19. Exclusion limit of the chloroplast pore. The ^3H_2O space was 49.6 μℓ/mg chlorophyll. (From Flügge, U. I. and Benz, R., *FEBS Lett.*, 169, 85, 1984. With permission.)

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