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Nonspecific and Specific Diffusion Channels in the Outer Membrane of Escherichia coli

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The outer membranes of Escherichia coli and Salmonella typhimurium contain several proteins that have been shown to produce rather large channels for diffusion [1]. One class, called "porins," has apparent molecular weights in the range of 34–37,000, and, when reconstituted into phospholipid-lipopolysaccharide bilayers, produced channels allowing the diffusion of sugars, sugar phosphates, nucleotides, amino acids, and polyethyleneglycols as long as their molecular weights were less than 600 [2, 3]. These results suggested, at least to us, that porin channels were largely nonspecific and acted as simple molecular sieves. Outer membrane proteins of another class, in contrast, appear to be involved in the transmembrane diffusion of special classes of solutes [1, 4]. At least one member of this group, the phage λ receptor protein (or *lamB* protein) of an apparent molecular weight of 50,000, was shown to produce transmembrane channels in reconstituted planar bilayers and vesicles [5, 6], and to function in the transport of oligosaccharides of maltose series in intact cells [7]. It is tempting to assume, therefore, that these proteins form specific channels quite different from the nonspecific, porin channels. However, reports from several laboratories have presented conclusions contrary to the hypotheses presented above, and suggested that porin channels had some solute specificity whereas the λ receptor channel was nonspecific [6–8]. The purpose of this short review is to critically analyze the results from other laboratories, and to summarize our recent data pertaining to the question of the specificity of protein channels in the outer membrane.

We should first define the word "specificity." Since diffusion through water-filled channel is a physicochemical process, it will obviously be influenced by the gross physicochemical properties of the solute, such as its size, hydrophobicity, and charge. Thus, if two solutes differing in some of these properties are tested, their diffusion rates are likely to be different. This does not indicate the presence of specificity. We use the word specificity in the sense traditionally used in biochemistry, ie, to mean configurational specificity or stereospecificity. Thus, a specific channel should discriminate between solutes that are similar in their gross physicochemical properties.

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ANALYSIS OF RESULTS FROM OTHER LABORATORIES

Are the Porin Channels Specific?

The presence of more than one species of porin in any given strain, and the finding that the biosynthesis of each of these species is controlled by different physiological conditions (summarized in [1]) suggested that each of these individual porins may have specialized transport functions. Several laboratories [8–11] have examined porin-facilitated diffusion in intact E coli cells lacking one of the two porin species. This was done by coupling the influx of solutes through the E coli outer membrane with their subsequent hydrolysis by periplasmic enzymes or with their active transport into the cytoplasm, and by measuring the overall rates of hydrolysis or transport. Sensitivity to various inhibitory agents was also investigated as a crude index of outer membrane permeability [9–11]. The results were quite consistent and included the following: a) The mutational loss of porin Ib did not produce detectable reduction in the overall rate of transport of any solutes tested [8–11]. b) The mutational loss of porin Ia did not produce a significant decrease in the rate of transport of various amino acids and sugars [9–11] but reduced the rates of transport and hydrolysis of nucleotides [8–11]. It also increased resistance to chloramphenicol, tetracycline, Cu^{2+} and Ag^{+} [9, 10, 12]. Mutants deficient in both porins Ia and Ib showed greatly decreased rates of transport of virtually any solutes tested [8–11]. d) However, even with these mutants the transport or hydrolysis rates were raised to a level comparable to those of the wild type if high external concentrations of solutes were used [8–11].

Various interpretations of these results have been presented, and some of them explicitly or indirectly invoked the concept that one or both of these pores were "specific." Thus it has been claimed [8], on the basis of observation (b), that Ia channel was specific for nucleotides which, nevertheless, could diffuse through alternative "nonspecific" channels according to the observation (d) above. Similarly, the observation (d) was used to argue that "other pores exist in the outer membrane," and that "at high concentrations of metabolites, diffusion through these secondary pores would be sufficient" [9]. Furthermore the observation (a), obtained with nucleotides, was interpreted to mean that Ib pores are specific for other substrates but did not allow the passage of nucleotides [8].

The problem in drawing these conclusions from such studies is that the *overall* rates of these coupled processes are determined not only by the permeability of the outer membrane but also by the V_{max} and K_m of the active transport process or the periplasmic hydrolase, but in these studies we cannot distinguish which is the rate-limiting step. Thus all of the observations described above can be explained very well by the mathematical analysis of the coupled two-step process, presented in detail in our recent review [13], and our current knowledge of the properties of the porin channel (see below). Observation (a) is expected because the magnitude of permeability (permeability coefficient) created by the presence of porin Ib is about one-tenth of that due to the presence of porin Ia for any substrate we have tested so far. None of the transport assays with intact cells could have detected 10% decrease in the permeability of the outer membrane. Observation (b) becomes understandable when we realize that reduction in the outer membrane permeability affects the overall rates of transport only when the former is the limiting step, a situation that may not exist with many substrates. With amino acids tested, the V_{max} of the active transport system is usually so low that even greatly reduced diffusion rates through the outer membrane are able to saturate the active transport process, and

we see little effect of the loss of porin Ia. For some sugars, the V_{max} of the active transport system is frequently high, but the system is still saturated by the diffusion through the outer membrane because monosaccharides have very high rates of diffusion through porin channels as they are small, very hydrophilic, and uncharged (see below). In contrast, the effect of loss of Ia is noticeable with nucleotides, because even in the wild-type cells, their slow rate of diffusion through the outer membrane, owing to their large size and negative charge, is the limiting step in the overall process of transport or hydrolysis. Finally, there is no need to assume the presence of "other pores" in order to explain the observation (d). All of the porin mutants appear to contain some residual porins, and the rate of diffusion through the porin channel increases in proportion to the external solute concentration according to Fick's first law. Therefore, at high external concentrations of solutes, the overall rates of transport or hydrolysis in porin-deficient mutants would approach those of the wild-type, as analyzed mathematically in our previous review [13].

In summary, none of the existing data proves or even strongly suggests the presence of specificity in porin channels. Furthermore, the data are totally compatible with our conclusion that Ia and Ib channels are nonspecific and very similar in their properties, except for the magnitude or permeability coefficients they produce (see below).

Are the λ Receptor Channels Nonspecific?

Recent investigations have raised doubts on the presumed specificity of the λ receptor channel. Reconstitution of the protein into planar phospholipid bilayers produced channels allowing the permeation of alkali metal cations [5], and reconstituted vesicles appeared to be permeable to various oligosaccharides structurally unrelated to maltose [6]. Thus, it has been proposed that this protein produced large, nonspecific channels, and the specificity seen in intact cells is conferred by the physical association of periplasmic maltose-binding protein with the channel [14, 15].

However, we feel that these studies did not properly answer the question of specificity. The planar bilayer experiments [5] measured the ion flux rates as conductivity, but the flux of the presumably favored solute, ie, maltose, could not be measured by this technique and hence the element of comparison is missing. The vesicle studies [6] measured the distribution of solutes after the long equilibrium. For a passive diffusion process, any solute within the exclusion limit of the channel will eventually diffuse out of reconstituted vesicles until it reaches equilibrium. Therefore, this method is not sufficiently sensitive to be able to detect preferences of the channel for certain solutes. For this purpose, the measurement of diffusion *rates* is essential.

PROPERTIES OF PORIN CHANNELS – INTACT CELL STUDIES

Effect of Solute Size

Since the existing data did not provide sufficient material for coming to decisive conclusions on the question of the specificity of channels, we performed a number of experiments first with intact cells, and then with reconstituted vesicles. In one series of experiments we measured rates of penetration of three sugars, arabinose, glucose, and lactose, which have the Stokes radius of 0.38, 0.42, and 0.54 nm, respectively [16]. For this purpose, we used E coli B/r, which produces only one species of porin resembling the porin Ia of E. coli K12 [17]. Furthermore, in order to make the outer membrane diffusion step a limiting one in the overall transport process, we used a strain containing a reg-

ulatory mutation, *ompB*, which reduces the number of porins per cell without altering the structure of the porin [18]. Under these conditions we could obtain the rates of diffusion of the sugars from their transport rates, which in turn were calculated from the growth rates of the mutant on various external concentration of these sugars [19]. The results showed that the rates of diffusion were strongly dependent on the size of the solute. Thus glucose diffused only at about one-half of the rate for arabinose, and the lactose flux rate was about 5% of the rate for glucose. This is as predicted by Renkin [20] for diffusion of solutes through small channels, and the use of the Renkin equation results in the estimation of a pore radius of about 0.6 nm.

Effect of Solute Hydrophobicity and Charge

For these experiments we introduced, into E coli strains producing either porin Ia alone or porin Ib alone, an R factor coding for a periplasmic β-lactamase. We then measured the rates of hydrolysis of various β-lactam antibiotics by intact cells, and at the same time measured the V_{max} and K_m of the enzyme by using sonicated cells. In this approach, pioneered by Zimmermann and Rosselet [21], one can adjust the external concentration of β-lactams so that the diffusion through the outer membrane would become a rate-limiting step in the overall process. Furthermore, by combining the rate equation of the outer membrane flux (governed by Fick's first law) with that of the hydrolysis in the periplasm (determined by Michaelis-Menten kinetics), one can calculate the actual values of permeability coefficient of the outer membrane toward various β-lactams [22].

The effect of hydrophobicity was tested by using monoanionic cephalosporins. Among these solutes of similar sizes (339–462 daltons) and charge, a tenfold increase in the octanol/water partition coefficient appeared to result in a fivefold reduction of permeability coefficient in both Ia and Ib channels. Thus another, gross, physicochemical property of the solute affected solute diffusion rate through porin channels, and it did so in a similar manner regardless of whether the channel was made of porin Ia or Ib. The only significant difference found between the two channels was that Ia-containing cells were about ten times more permeable than Ib-containing cells toward any given solute [22]. The number of porin molecules per cell is not so different between these two strains, and thus one possible explanation is that most of the Ia channels are "open," whereas most of the Ib channels are "closed" under our conditions of assay. We are attracted to this hypothesis in view of Schindler and Rosenbusch's observation [23] that application of membrane potential of more than a certain value "closes" the porin channel reconstituted into planar lipid bilayer films.

The use of other kinds of cephalosporins showed that the presence of additional negative charge on the solute produced a strong decrease in permeability coefficient. Furthermore, Ia and Ib channels were again affected in exactly the same manner. Thus, cefsulodine, which is essentially cephaloridine carrying an added SO_3^- group, diffused 30 times more slowly than cephaloridine through porin Ia channel, and 21 times more slowly through porin Ib channel [22]. In conclusion we could not find any difference in the ways the penetration rates in Ia and Ib channels are affected by the gross physicochemical properties of the solutes.

PROPERTIES OF PORIN AND λ RECEPTOR CHANNELS STUDIED WITH RECONSTITUTED VESICLES

In intact cell studies, we are limited to certain kinds of solutes. Moreover, the possibility that the solutes are crossing the outer membrane via channels other than those of our interest cannot be completely excluded. This is a serious drawback especially in

studies of specific channels, although it is unlikely to be a problem with porin, which has been shown to be responsible for most of the nonspecific diffusion processes of the outer membrane [24, 25]. A further problem in the case of λ receptor channel is that with intact cells we cannot separate the functions of the outer membrane channel from those of the periplasmic binding protein.

These considerations prompted us to find an assay for solute flux rates usable in reconstituted vesicles containing only the purified channel-forming protein. As already mentioned, conductivity assay with planar bilayers measures only the flux of ionized compounds, and vesicle assay involving filtration of vesicles [2, 3, 6] only gives data reflecting the near-equilibrium distribution of solutes. In our new assay [26] we utilize the technique widely used for measuring the permeability of non-protein-containing liposomes toward various solutes [27]. We prepare multilayered phospholipid vesicles with channel-forming proteins incorporated into the bilayers and dilute them in isotonic solutions of the test solute. The *rate* of penetration of the solute through the outermost layer of the vesicle is calculated from the initial rate of swelling of the vesicles, as determined by the rate of change of turbidity of the suspension. The vesicles are preloaded with solutes which could not diffuse across the membrane, and we use 20,000-dalton dextran which cannot penetrate through even the largest porin channels known [28, 29].

When these "swelling" assays were performed with vesicles containing porin Ia or λ receptor protein from E coli K12, results shown in Figure 1 were obtained with hexoses and hexitols. With the porin channel, the flux rates of hexoses were quite similar. Hexitols diffused somewhat faster than hexoses, presumably because of the flexibility of their open-

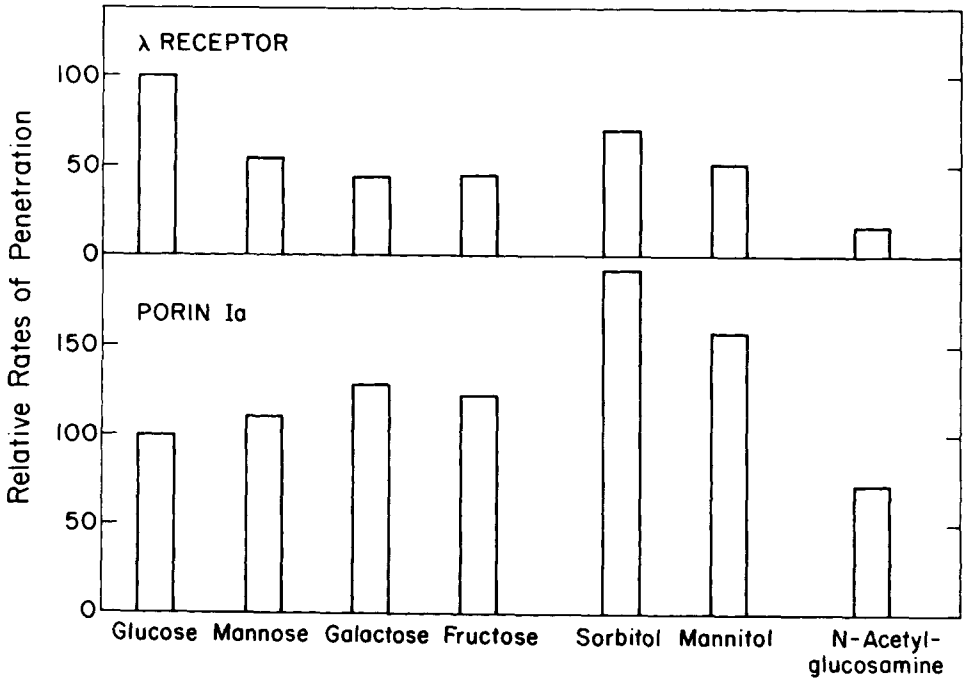

Fig. 1. Rates of diffusion of hexoses and hexitols into liposomes containing purified λ receptor protein or purified porin Ia. The rates were calculated from the swelling rates of liposomes as described previously [26]. The data for λ receptor channels are taken from [26]. The data for porin Ia were taken from our study to be published [22]; liposomes contained 0.17 μg of the porin per mg of phospholipids. The penetration rate for glucose was arbitrarily set as 100.

chain conformation. N-acetylglucosamine diffused significantly more slowly, probably owing to the presence of a bulky acetylamino side chain. Thus, to a first approximation, the diffusion rates through the porin channel can be correlated with the size of the solute in solution.

With the λ receptor channel, however, the rates of solute diffusion seem to be affected not only by the gross physicochemical properties but also by the specific configuration of the solute. Glucose is very strongly favored among the four hexoses with similar physicochemical properties, and sorbitol (ie, glucitol), which should have a more flexible conformation than glucose, actually diffused more slowly than glucose.

The configurational specificity of λ receptor channel becomes even more apparent when we examine the diffusion rates of disaccharides (Fig. 2). Thus, among disaccharides which share reasonably similar physicochemical properties, there was a strong discrimination so that sucrose diffused at a rate only about 1/40 of that of maltose. In contrast, with the porin channel the maximal difference in diffusion rates among disaccharides was only two- to threefold. (This difference is more pronounced than that seen with monosaccharides, but this is expected because disaccharides are much closer to the exclusion limit of the channel, and the degree of retardation would be more dependent on small differences in solute size and shape.)

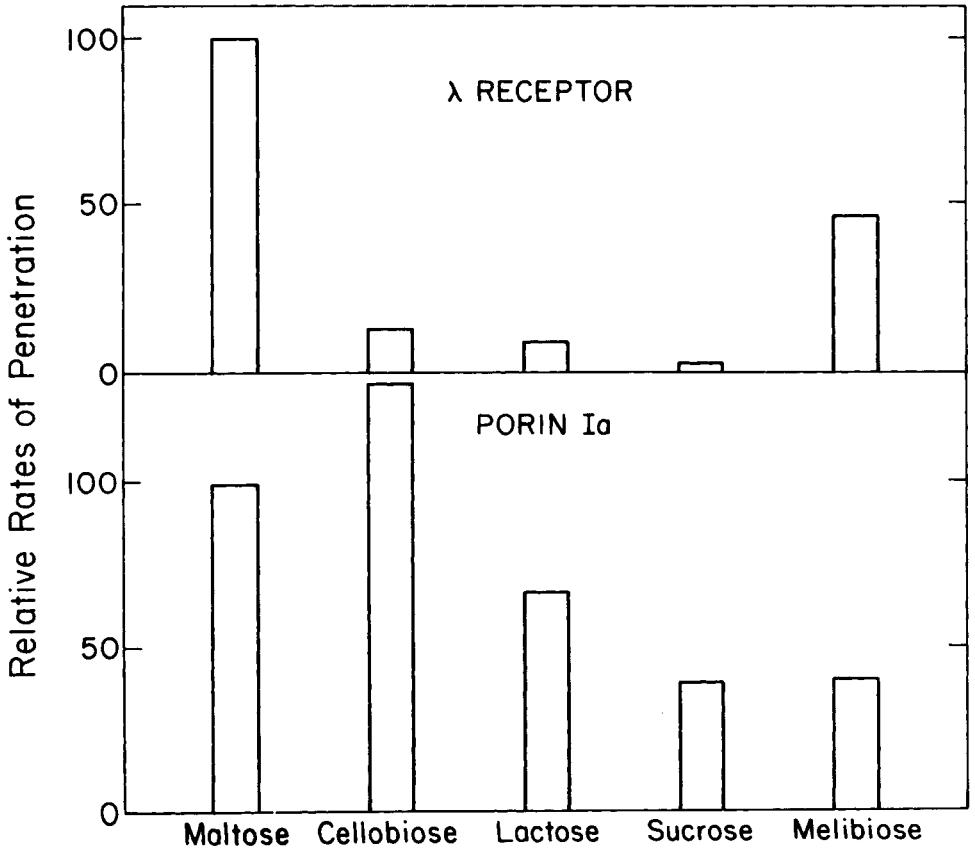

Fig. 2. Rates of diffusion of disaccharides. Liposomes used for the assay of the permeability of the porin channel contained 6 μg of the protein per mg of phospholipids [22]. For other details, see Figure 1. The penetration rate of maltose was arbitrarily set as 100.

When we used higher oligosaccharides, we found that the *rate* of diffusion of trisaccharides through the porin channel was extremely slow (less than 0.5% of the rate for glucose), although in Nakae's assay [2, 3], which uses far larger amounts of porin, most of the raffinose trapped inside did diffuse out of the vesicles during the long gel filtration (or Millipore filtration and washing) step. The λ receptor channel, in contrast, allowed the diffusion of the heptasaccharide of the maltose series at a significant rate (2.5% of the rate of maltose flux). For these higher oligosaccharides the channel was virtually specific for oligosaccharides of maltose series.

CONCLUSIONS

Studies with intact cells, equilibrium permeation assay with vesicles according to the procedure of Nakae [2, 3], and more recent flux rate assay with vesicles all indicate that porin channels act as a nonspecific, molecular-sieving channel. Its diameter can be estimated in two ways. First, the Stokes radius of the largest molecule that can penetrate the channel, ie, raffinose, is 6.0 Å [17], and this observation suggests a diameter of 12 Å. Secondly, from the rates of diffusion of solutes of various sizes one also obtains the diameter of 12 Å by the application of the so-called Renkin equation (see above). It is gratifying to note the agreement between these figures, and to note further that negative staining of the two-dimensional crystals of E coli porin revealed indentations with a diameter in the neighborhood of 15–20 Å [30].

It is impossible to prove rigorously the absence of any configurational specificity. However, most of the flux rate data with the porin channel can be explained on the basis of the gross physicochemical properties of the solute. Penetration of the larger solutes is hindered because they frequently collide with the rim and fail to enter the pore, and also because they experience stronger viscous drag from the wall of the pore. The diffusion of the hydrophobic compounds is retarded by a large energy barrier, presumably because these solutes have to break many hydrogen bonds in the channel and cannot replace them with new hydrogen bonds between the solute and groups on the channel wall. The retardation of negatively charged solutes may be due to the presence of many anionic groups on the channel wall; it is interesting to recall that porins are the most acidic proteins present in the outer membrane.

In contrast to porin, the λ receptor channel clearly exerted discrimination of solutes on the basis of their configuration. This is "specificity" in the sense used customarily in biochemical literature. Obviously this stereospecific discrimination cannot be achieved without specific solute-binding sites, and it is reassuring that higher oligosaccharides of the maltose series were found to bind to these sites with an apparent K_i of the order of 1 mM [31]. It is also gratifying to know that the binding of α (1 → 4)-linked glucan chains to the λ receptor protein has been demonstrated directly by the use of a fluorescent derivative of amylopectin [32].

One important feature of the λ receptor channel is that it acts like a nonspecific, porin-like channel for very small solutes, allowing their passage regardless of their structural similarity (or lack of it) to maltose. Thus in reconstituted systems, alkali metal cations [5], Tris [26], and serine [26] have been found to penetrate rapidly through this channel. These results suggest that penetration does not always require the binding of the solute to the specific binding site. The situation is obviously quite different from substrate-enzyme interaction, in which the substrate must bind to the enzyme in order to become activated.

The rates of passage of solutes structurally unrelated to maltose should in principle enable us to estimate the size of this "nonspecific" portion of the channel. At present this is difficult because most of the solutes we tested are sugars that have some common

structural features with maltose. However, if we choose arbitrarily galactose and lactose as the "unrelated" solutes, their relative rates of penetration suggest, on the basis of Renkin equation, an "effective pore size" of the same order of magnitude with that of porin Ia channel.

In view of the apparent narrowness of this "nonspecific" part of the channel, it is all the more impressive that the λ receptor protein can facilitate the diffusion of very long oligosaccharide chains, up to maltoheptaose in our experiment and to at least maltopentadecaose in intact cell experiments by Ferenci [33]. It seems most likely that the diffusion of these higher oligosaccharides is preceded by their binding to a specific site within the channel, and the binding causes the oligosaccharide to be aligned in a correct conformation, so that their gradual penetration can now take place (see Fig 3). The looseness of the binding site(s) would enable movement of the solute, possibly driven by only the chemical potential difference across the membrane. However, especially with long oligosaccharides, the presence of high affinity binding proteins on the periplasmic side will accelerate the process significantly.

The λ receptor channel thus behaves as a specific channel. However, it is possible that intact cells contain additional mechanism(s) to increase the specificity further. In fact, although the isolated λ receptor protein allows a rapid diffusion of glucose, intact cells apparently take up glucose only very slowly through the λ receptor channel [34]. It is unclear at present whether this is due to alteration of the channel during isolation, or to the physical association of λ receptor protein with maltose-binding protein, proposed by several workers [14, 15].

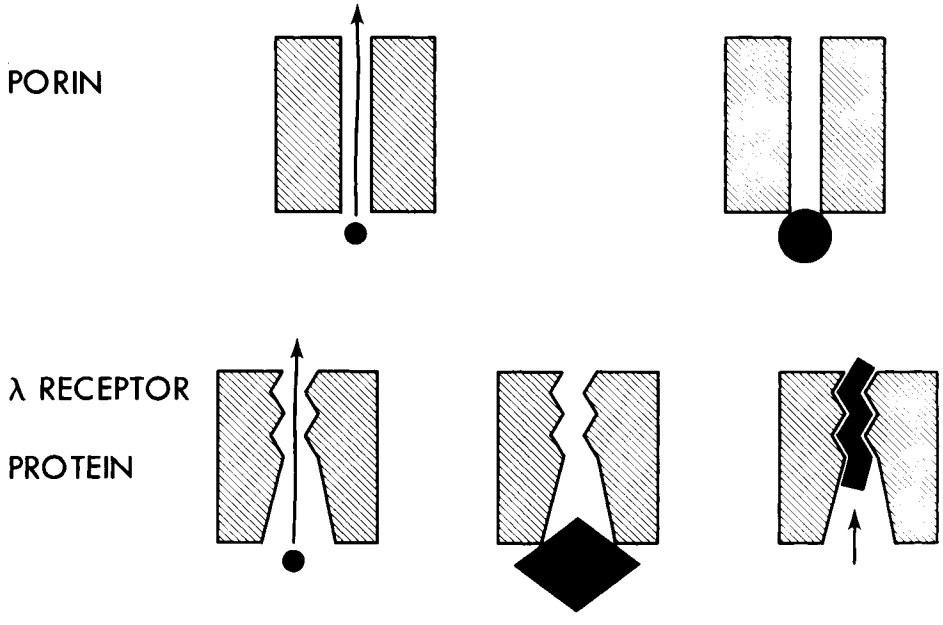

Fig. 3. Speculative models of porin and λ receptor channels. Porin channels are conceived essentially as cylindrical channels with no specific combining sites. The λ receptor channel allows the nonspecific penetration of very small solutes (lower left) or the binding and subsequent penetration of the oligosaccharides of the maltose series (lower right), but excludes larger molecules that cannot fit into the binding site (bottom center).

We have seen that the λ receptor protein behaves as a transmembrane channel with solute specificity, whereas the porin channels operate as nonspecific pores. Similar protein channels probably form the central portion of active transport complexes, "gates," and "pumps" in numerous biomembranes. We feel that the studies of these rather simple channels in the bacterial outer membrane will contribute significantly toward our understanding of the mechanisms of transmembrane flux processes.

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