Outer Membrane as a Diffusion Barrier in *Salmonella typhimurium*

**Penetration of Oligo- and Polysaccharides into Isolated Outer Membrane Vesicles and Cells with Degraded Peptidoglycan Layer**

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In *Escherichia coli* and *Salmonella typhimurium*, the cell wall that contains both the outer membrane layer and the peptidoglycan layer acts as a barrier of the molecular sieve type for the penetration of uncharged saccharides (G. Decad, T. Nakae, and H. Nikaido (1974) *Fed. Proc. 33*, 1240). Here we examined which of the layers of the cell wall limited the size of the penetrating molecules, by studying the penetration of saccharides into (a) cells whose peptidoglycan layer had been destroyed by lysozyme treatment or growth in the presence of penicillin and (b) isolated outer membrane vesicles. We found that peptidoglycan-defective cells were similar to intact, plasmolyzed cells in that they allowed a partial penetration of stachyose (molecular weight 666), but essentially excluded saccharides with molecular weights higher than 900 to 1000. We also found that the isolated outer membrane acted as a penetration barrier for saccharides. These observations led us to conclude that the outer membrane, rather than peptidoglycan, sets the size limit for the penetration of uncharged, hydrophilic molecules through the *E. coli* or *S. typhimurium* cell wall. The isolated outer membrane, however, had an exclusion limit much higher than that found in intact cells. This "leakiness" could be decreased either by the use of mutants producing extremely deficient lipopolysaccharide, or by trypsin treatment of the isolated membrane followed by heating and slow cooling in the presence of Mg++. We feel that these observations are consistent with the hypothesis that the resealing of the ruptured outer membrane during the isolation procedure is often incomplete, and that cracks and holes thus generated are responsible for the "leakiness" of the isolated membrane vesicles.

Gram-negative bacteria such as *Escherichia coli* or *Salmonella typhimurium* are covered by a double membrane system (3, 4). The inner, or cytoplasmic membrane is surrounded by the "outer membrane," and the peptidoglycan layer is located between these two membranes. Since the components of known active transport systems are usually found in the cytoplasmic membrane (5, 6), the outer membrane had been thought as a rather inert structure that allows the penetration of all low molecular weight substances.

Increasing numbers of evidence that have accumulated in recent years suggest, however, that the outer membrane indeed constitutes a barrier for the penetration of certain kinds of molecules. The relevant data include the following: (a) *E. coli* and *Salmonella* mutants defective in the biosynthesis of lipoplysaccharide, a component of the outer membrane, were found to be more sensitive to various antibiotics, dyes, and bile salts (7-12), and to allow the more rapid penetration of a dye, crystal violet (13). (b) Short exposure of *E. coli* or *Salmonella* to EDTA was found to release about one-half of the lipopolysaccharide from the outer membrane of the cells and at the same time make the cells sensitive to antibiotics to which they are normally resistant (14-17). (c) Kinetics of β-thiogalactoside exit from *E. coli* suggested the presence of a partial diffusion barrier outside the cytoplasmic membrane (18). (d) *E. coli* cannot utilize peptides larger than a certain size limit, a finding which is consistent with the existence of a molecular sieving barrier on its surface (19).

In spite of the presence of these many pieces of indirect evidence, no direct study of outer membrane permeability has been reported. Therefore, we have examined the penetration of oligosaccharides across the cell wall (i.e. outer membrane plus peptidoglycan) of plasmolyzed *E. coli* and *S. typhimurium* cells, and found that the cell wall constitutes a significant diffusion barrier for a molecule as small as stachyose (666 daltons) (2). This paper extends these oligo- and polysaccharide penetration studies to two new systems: (a) isolated outer membrane vesicles, and (b) cells with degraded peptidoglycan layer. The results suggest that the outer membrane layer, rather than peptidoglycan layer, sets the size limit for the penetration of saccharides through cell wall.
**Experimental Procedure**

**Bacterial Strains—Salmonella typhimurium** LT2 and its mutants were used. The latter, HN202 (Escherichia coli K-12, M-3 of Ref. 20) and TA2168 (hisC076 gaiE506 rfa-l009) (22) produce incomplete lipopolysaccharides of Rc and Re types (22), respectively.

**Isolation of Outer Membrane Vesicles—Bacteria were grown and the outer membrane vesicles were isolated according to the procedure of Osborn and co-workers (23), except that the spheroplasts were lysed by slowly pouring the spheroplast suspension into 5 to 10 volumes of ice-cold distilled water containing pancreatic deoxyribonuclease (Sigma, type D) and pancreatic ribonuclease (Sigma, type XII) (10 μg/ml). With TA2168 the separation of the outer membrane band from the intermediate band ("M band") was insufficient; we therefore collected carefully only the leading portion of the former band. Such a preparation contained less than 5% of inner membrane, as determined by DPNH oxidase assay (23).

For permeability experiments, the vesicles were used immediately after their preparation. Less than 2% of the total phospholipids in these preparations correspond to lysophosphatidylethanolamine.

**Determination of Outer Membrane Permeability—**The standard procedure to isolate the outer membrane was as follows. To an appropriate buffer solution containing membrane vesicles (containing 3 to 5 mg of protein), $[^{14}C]$glycerol (or $[^{14}C]$sucrose) (0.1 to 0.15 μCi), and $[^{3}H]$oligosaccharide (or $[^{3}H]$julin or $[^{3}H]$dextran) (0.3 to 0.45 μCi) was added. The reaction mixture was rapidly agitated by slow pouring the spheroplast suspension into 5 to 10 volumes of ice-cold distilled water containing pancreatic deoxyribonuclease (Sigma, type D10 (10 μg/ml)) and pancreatic ribonuclease (Sigma, type XIIIB) (10 μg/ml). With TA2168 the separation of the outer membrane band from the intermediate band ("M band") was insufficient; we therefore collected carefully only the leading portion of the former band. Such a preparation contained less than 5% of inner membrane, as determined by DPNH oxidase assay (23).

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**Penicillin Spheroplasts—**An exponential phase culture of HN202 in 10 ml of medium M-9 (26) was diluted 4-fold with Difco antibiotics medium No. 3 containing 20% sucrose, 0.2% MgSO$_4$, and 0.1% penicillin G, prewarmed to 37°C. The 6-liter Erlenmeyer flask containing the suspension was shaken at 40 rpm, shaken at 37°C on a water bath shaker (New Brunswick, model G-77) for 2 hours, and the spheroplasts were collected by centrifugation (2000 × g for 10 min) and were suspended in 4 ml of appropriate buffer solution containing membrane vesicles, (b) centrifugation was at 80 x g rather than at 25,800 x g, (c) centrifugation was at 10 min instead of 100 min, and finally resuspended in 4 to 6 ml of 0.75 M sucrose-0.1 M Tris-HCl (pH 7.5)-100 μM of lysozyme. Although most cells still contained a cell wall, those that did not were broken up by the addition of radioactive compounds to the vesicles, the buffer usually contained nonradioactive sucrose and glycerol at the final concentrations of 0.25 and 0.01 M. In some experiments, nonradioactive oligosaccharide, julin, or dextran was also added, but this did not alter the results.

The reaction mixture (final volume, 0.50 ml) in 1.5-ml plastic centrifuge tubes (Eppendorf, No. 3810) was mixed immediately for 5 s with a Vortex-type mixer, and then was kept at room temperature for 10 min without further agitation. The tubes were centrifuged at room temperature in a SS-34 rotor (equipped with rubber adapters, Catalogue No. 317, Ivan Sorvall) of a Sorvall RC-2B centrifuge for 10 min at 10,000 x g. The tubes were centrifuged again as before. The supernatant (500 μl) was transferred to separate vials, and the radioactivity was determined.

**Permeable space (μl) = total radioactivity in second supernatant − radioactivity/m of first supernatant × radioactivity in 50 μl of second supernatant

The permeable space (μl), however, was affected by the completeness of the removal of the first supernatant; variations of ±5% were seen among similar samples. In order to circumvent this difficulty, we took advantage of the double label technique. Usually our $[^{14}C]$ labeled compounds could penetrate freely through the outer membrane (2) (see also "Results"). Thus the permeable space for $[^{14}C]$ compounds would be a sum of (a) intravesicular space, (b) intervesicular space, and (c) volume of the supernatant that could not be removed. If the $[^{14}C]$ labeled compound used was completely impermeable through the membrane, the permeable space for the $[^{14}C]$ compound will be the sum of b and c above. If we take the difference (permeable space for $[^{14}C]$ compound) – (permeable space for $[^{3}H]$ compound), the result would be equal to a, or intervesicular space, and this is not affected by the completeness of removal of the first supernatant. We therefore routinely calculated this difference, "$[^{3}H]$-impermeable space," which is equal to intervesicular space for nonpenetrating compounds, close to zero for fully permeable compounds, and in between for partially penetrating compounds.

For reasons described under "Results" we calculate the intervesicular space by using 16,600 daltons dextran, which we assume to be completely impermeable through the outer membrane. The degree of penetration for any other $[^{14}C]$-labeled compound is given by:

$$1 - \text{"}[^{3}H]\text{-impermeable space for that compound } \times 100$$

\[ \text{intravesicular space} \]

**Plasmolysed Cells—**HN202 cells were grown in L broth (24) (glucose omitted), (1,500 ml in 6-liter Erlenmeyer flask) at 37°C with vigorous shaking (300 rpm, New Brunswick gyrotary shaker model G-77), and then were harvested by centrifugation at 6,000 × g for 5 min. (This as well as all subsequent operations were carried out at room temperature.) The cells were washed once with 0.1 M NaCl, and suspended in 4 to 6 ml of 0.1 M NaCl. The penetration of saccharides was tested in a manner similar to that described above for the outer membrane vesicles, except that (a) cells (30 to 50 mg wet weight) were used instead of membrane vesicles, (b) centrifugation was at 80 × g, rather than at 25,800 × g, and (c) NaCl was added to the reaction mixture to a final concentration of 0.5 M in order to produce plasmolysis. $[^{14}C]$Sucrose was used as the compound that penetrates through the cell wall but not through the inner, cytoplasmic membrane. The "$[^{3}H]$-impermeable space" in this experiment was related to the size of, and the degree of penetration of $[^{3}H]$-saccharides into the periplasmic space.

**Fractionation of [methoxy-$[^{3}H]$]dextran—**About 75 μCi of [methoxy-$[^{3}H]$]dextran (New England Nuclear Corp., NET-427A, Lot 3256, specific activity 1 μCi/mg) were applied to a column (2.54 x 51 cm) of Bio-Gel P-30, and Bio-Gel P-200 mesh (Bio-Rad Laboratories, Richmond, Calif.), which was eluted with 0.1 M NaCl. The highest peak of radioactivity was found in fractions eluted shortly after the void volume. However, significant amounts of radioactivity were found in almost all fractions ranging from the void volume to the complete inclusion volume. Neighboring fractions were pooled and purified by repeating gel filtration usually twice on Bio-Gel P-30, Bio-Gel P-10, or both. The "peak" zone was collected, dried, and dialyzed against 0.1 M NaCl. The radioactivity in the fractions eluting within the peak was then collected, lyophilized, and partitioned into three fractions on a column (1.7 x 85 cm) of Bio-Gel A-0.5 m (200 to 400 mesh, Bio-Rad), which was eluted with 0.1 M NaCl-0.2% NaN$_3$. These two $[^{3}H]$dextran fractions were eluted at
the time when 19% and 44% of the Dextran T10 (determined by phenol-sulfuric acid reaction, therefore, by weight) had been eluted. From the molecular weight distribution of this lot of Dextran T10, supplied by the manufacturer, the molecular weights of the two [3H]dextran fractions were determined to be 16,500 and 10,000.

All [3H]dextran and [3H]inulin fractions as well as stachyose and raffinose were then applied, in several combinations, to a column (1.27 x 95.7 cm) of Sephadex G-50 (fine, Lot No. 2961), eluted with 0.1 M NaCl containing 0.02% NaN3. Then by using the two [3H]dextran fractions described above, stachyose, and raffinose as molecular weight standards, the molecular weights of other [3H]-polysaccharides were determined graphically (Fig. 2). Although dextran standards of known molecular weights were not available in the intermediate range, this procedure was justifiable in view of the fact that a plot of published $K_{av}$ values of dextrans in Sephadex G-50 (27) against logarithms of molecular weights yields a perfect straight line (not shown).

Another fraction of [3H]dextran was obtained by fractioning [methoxy-3H]dextran from New England Nuclear (Catalogue No. 427-B; Lot No. 622-221; specific activity, 1 mCi/18.7 mg) on a column (1.27 x 50 cm) of Bio-Gel P-150. Fractions with the $K_{av}$ values of 0.05 to 0.2 were pooled and used as “100,000-dalton [3H]dextran.”

Other Methods—Protein was determined with phenol reagent (28), carboxylate by phenol-sulfuric acid reaction (29). Bacterial growth was monitored with a Klett-Summerson colorimeter with a red filter.

Radioactivity was determined with a Nuclear-Chicago Isocap 300 liquid scintillation spectrometer using windows manually set for double label counting. Ten milliliters of Bray's solution (30) was added to each vial. The spillover of the counts into the opposite channel was usually less than 8%. In order to correct for spillover, blanks containing all ingredients of the reaction mixture except the radioactive markers were always run in parallel, and these blanks were counted after the addition of [3H]- or [14C]bead.

Electron Microscopy—Samples were prepared for transmission electron microscopy, using the method described (29). Indirect immunofluorescence was carried out by Dr. W. Smit of this laboratory. The sections of the outer membrane, examined by transmission electron microscopy, gave images very similar to the pictures obtained by Osborn and her co-workers (23).

Sections of the outer membrane, examined by transmission electron microscopy, gave images very similar to the pictures obtained by Osborn and her co-workers (23). More than 90% of the membrane fragments appeared to be closed vesicles. There were about equal numbers of large vesicles (diameter 0.2 to 0.5 pm) and small vesicles (diameter 0.02 to 0.05 pm, in some cases as large as 0.1 pm).

The closed vesicular structure of the isolated outer membranes was confirmed by scanning electron microscopy. This technique revealed many vesicles with almost perfectly spherical shape. The surface of the vesicles appeared smooth and structureless, and no obvious cracks or holes could be detected except in a few vesicles which were deformed presumably during the preparation of the specimen.

The closed nature of the vesicles was also confirmed by equilibrium centrifugation in density gradients. It is known that closed membrane vesicles equilibrate with solutions of different densities depending on the penetrability and the osmotic activity of the solute used for making the gradients (31). If the solute is impermeable through the membrane and has negligible osmotic activity as is true for Ficoll (Pharmacia), for example, the vesicles come to equilibrium at a position at which the density of vesicles with their intravesicular water (which does not contain Ficoll) is identical with the density of the surrounding solution. In contrast, if the solute penetrates freely into the intravesicular space, the vesicles will come to an equilibrium at a position where the density of the surrounding medium is equal to that of the membrane layer itself, which would be much higher than the buoyant density obtained in Ficoll gradient. We therefore centrifuged an outer membrane preparation in 30 to 60% gradients of Ficoll and sucrose for 20 hours at 159,200 x g in an SW 65K rotor of Spinco L-2 centrifuge. The apparent buoyant density of the outer membrane was 1.178 and 1.179 (in two experiments) in Ficoll,

RESULTS

Isolated Outer Membrane Exist as Closed Vesicles—We tried to examine the permeability properties of isolated outer membrane vesicles, because we wanted to know whether the outer membrane layer is the main molecular sieving barrier in Salmonella cell wall. Since the method we use for permeability study requires that the membranes exist as closed vesicles, we first examined whether such a condition was fulfilled.
whereas a much higher density, 1.256 and 1.265, was found in sucrose. This result is consistent with the assumption that the majority of outer membrane fragments exist as closed vesicles which are permeable to sucrose but not to Ficoll (average $M_r$ 400,000).

Penetration of Saccharides into Outer Membrane Vesicles—[$^3H$]Dextran of various size (see “Experimental Procedure”) and [$^{14}C$]glycerol or [$^{14}C$]sucrose were added to thick suspensions of the outer membrane vesicles. After 10 min incubation at room temperature, the suspension was centrifuged, the pellet was resuspended in water after the careful removal of the supernatant, and the suspension was centrifuged again. The $^3H$ and $^{14}C$ concentrations in the first and the second supernatants were then used to determine the $^3H$-impermeable space in vesicles, as described under “Experimental Procedure.”

For example, outer membrane vesicles from HN202 (3.3 mg of protein), [$^3H$]dextrans (16,500 daltons), and [$^{14}C$]glycerol were mixed together in TSE buffer (10 mM Tris-Cl (pH 7.5)/0.25 M sucrose/1 mM EDTA) in a final volume of 0.5 ml. The outer membrane used should have contained lipopolysaccharide, phospholipid, and protein in the weight ratio of 0.3/0.3/1.0 (23). Thus the total weight of the membranes used would be $1.6 \times 3.3 = 5.3$ mg and the collapsed membrane would only occupy a volume of $5.3 \times 1.36 = 4.2 \mu l$, since the buoyant density of our outer membrane preparation was 1.26. In contrast, the centrifugation procedure gave a [$^{14}C$]glycerol-permeable space of 22.3 $\mu l$. This figure is much larger than the calculated space for collapsed membranes, and suggest that much space exists between the membranes. The following lines of evidence indicate that the large [$^{14}C$]glycerol-permeable space is not a result of adsorption of [$^{14}C$]glycerol to membranes. (a) The same [$^{14}C$]-permeable space was obtained regardless of the concentrations of carrier nonradioactive glycerol used, and regardless of the nature of [$^{14}C$]-labeled small molecules used (e.g. [$^{14}C$]glucose or sucrose instead of [$^{14}C$]glycerol). (b) The pellet actually weighed 28 mg, which is quite close to what is expected (22.3 mg of water between the membranes + 5.3 mg of membranes - 27.6 mg).

The same experiment gave a [$^3H$]dextran-permeable space of 10.4 $\mu l$. This space did not increase significantly when [$^3H$]dextran of larger molecular weight (100,000) was used instead of the 16,500-dalton dextran. We therefore assume that the 16,500-dalton dextran is excluded almost completely from closed vesicles, and that the space impermeable to the 16,500-dalton [$^3H$]dextran corresponds to the intravesicular space in closed outer membrane vesicles. Thus in this case, about 35% of the pellet volume (i.e. $10.4 \div 28 \times 100$) corresponds to intervesicular space, and about 43% of the pellet volume (i.e. $[22.3 - 10.4] \div 28 \times 100$) corresponds to intravesicular space. Similar figures have reproducibly been obtained for different outer membrane preparations from HN202.

When [$^3H$]dextrans of lower molecular weight were used, [$^3H$]dextran space obtained was larger than with the 16,500-dalton [$^3H$]dextran. We believe that this is due to the partial penetration of [$^3H$]dextrans into intravesicular space, and calculate the degree of penetration of intravesicular space as described under “Experimental Procedure” (Fig. 3).

The partial penetration can be the result of either the slow penetration into a uniform population of vesicles or complete penetration into only a portion of a heterogenous population of vesicles. A partially penetrating [$^3H$]inulin (2000 daltons) was incubated with outer membrane vesicles from HN202 and portions of the suspension (each containing 1.6 mg of membrane protein) were centrifuged at 10, 25, and 40 min. The [$^3H$]-impermeable space obtained was 3.1, 3.0, and 2.7 $\mu l$ for these samples, and clearly the penetration reached the near-maximal level already after 10-min incubation. We thus performed all the other centrifugation experiments with 10-min incubation. The results also suggest that the partial penetration is mainly due to the heterogeneity of the vesicle population.

When the outer membrane preparations from three Salmonella strains were incubated with [$^3H$]dextrans of varying size, considerable penetration into intravesicular space was seen with [$^3H$]dextrans of molecular weight below 10,000 (Fig. 3). The membrane from an Rc mutant (HN202) behaved very similarly to the membrane from the wild type (LT2), whereas the vesicles from the Re mutant (TA2168) appeared to be less leaky than the vesicles from the other two strains. We shall deal with this observation under “Discussion.”

Attempts to Decrease Leakiness of Isolated Vesicles—Since the isolated outer membrane vesicles allowed the penetration of much larger molecules than the outer membrane of plasmolysed cells did, we tried to find conditions which would produce vesicles with permeability similar to that found in intact cells.

We found that preincubation of the vesicles with 0.05 M MgCl$_2$ had a slight but reproducible effect in reducing the penetration of [$^3H$]dextran (not shown). Furthermore, heating and slow cooling in TSE buffer containing 0.05 M MgCl$_2$0.14 M NaCl gave vesicles which were decreased substantially in leakiness (Fig. 4). The best and most reproducible results, however, were obtained when the vesicles were first treated with trypsin, and then were heated to 60° and slowly cooled in TSE containing 0.05 M MgCl$_2$0.14 M NaCl (Fig. 4). The vesicles thus treated were essentially impermeable to [$^3H$]dextrans of molecular weight higher than 2,000, but they were still slightly more permeable to oligosaccharides than intact cells. Thus the 920-dalton [$^3H$]inulin penetrated to about one-half of
Preparations (circles, "Experimental Procedure.") -Labeled compounds used and their molecular weights (in parentheses) were: raffinose (504), stachyose (666), inulin (920), inulin (1,380), dextran (1,800), inulin (2,000), dextran (3,300), dextran (6,300), dextran (10,000), and dextran (16,500). Untreated outer membranes (circles) were tested in TSE buffer. Portions of the outer membrane preparation (8 to 10 mg protein/ml) were also treated with trypsin (250 μg/ml) for 1 hour at 37°C in TSE buffer containing 0.1 mg/ml of chloramphenicol, MgCl₂, and NaCl. Three experiments with different membrane preparations were established.

Fig. 4. Penetration of oligo- and polysaccharides into outer membrane vesicles and plasmolyzed cells of HN202. Outer membrane preparations (circles, crosses, and triangles) and cells plasmolyzed in 0.5 M NaCl (squares) were tested for penetration as described under "Experimental Procedure." ²H-Labeled compounds used and their molecular weights (in parentheses) were: raffinose (604), stachyose (666), inulin (920), inulin (1,380), dextran (1,800), inulin (2,000), dextran (3,300), dextran (6,300), dextran (10,000), and dextran (16,500). Untreated outer membranes (circles) were tested in TSE buffer. Portions of the outer membrane preparation (8 to 10 mg protein/ml) were also treated with trypsin (250 μg/ml) for 1 hour at 37°C in TSE buffer containing 0.1 mg/ml of chloramphenicol, MgCl₂, and NaCl. Three experiments with different membrane preparations were established.

Procedures which did not significantly decrease the leakiness of vesicles include the following: (a) trypsin treatment alone, (b) trypsin treatment followed by heating and slow cooling in TSE buffer, and (c) heating and slow cooling in TSE buffer. Procedures which did not significantly decrease the leakiness of vesicles include the following: (a) trypsin treatment alone, (b) trypsin treatment followed by heating and slow cooling in TSE buffer, and (c) heating and slow cooling in TSE buffer.

Permeability of Cells with Degraded Peptidoglycan Layers

—Another system we used for investigating the role of peptidoglycan and outer membrane in saccharide penetration was the cell wall. When we consider Hypotheses b and c above, we should keep in mind the fact that the peptidoglycan-rich cell wall of Gram-positive bacteria was shown to act as a molecular sieve-type barrier for the penetration of oligosaccharides (2). In that study, however, the precise location of the barrier has not been established.

Thus the barrier properties could be due to the presence of (a) peptidoglycan layer, (b) outer membrane, or (c) both of these layers. We have found in this study that the isolated outer membrane, which are largely devoid of peptidoglycan, did act as a diffusion barrier. This observation clearly rules out the Hypothesis a above, that peptidoglycan is entirely responsible for the barrier properties of E. coli and Salmonella cell wall.

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Thus the barrier properties could be due to the presence of (a) peptidoglycan layer, (b) outer membrane, or (c) both of these layers. We have found in this study that the isolated outer membrane, which are largely devoid of peptidoglycan, did act as a diffusion barrier. This observation clearly rules out the Hypothesis a above, that peptidoglycan is entirely responsible for the barrier properties of E. coli and Salmonella cell wall.

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can, the destruction of peptidoglycan could conceivably lead to gross outer membrane damages in a fraction of the population; this will then produce the biphasic per cent penetration versus molecular weight plots observed (Fig. 5).

What produces damages in the membranes of the isolated outer membrane vesicles? We have no unequivocal answer, but the following hypothesis seems to fit our experimental results well. When the outer membranes are ruptured during preparation, they usually reseal and form closed vesicles like any other biological membrane. There are two factors, however, which could make the resealing process unusually difficult for the outer membrane. (a) The outer membrane contains large amounts of lipopolysaccharide which has several anionic groups (22). Thus when the edges of the membrane fragment come close during the resealing process, there could be a strong electrostatic repulsion between lipopolysaccharide molecules. The repulsion is enhanced because most divalent cations must have been removed by EDTA during the preparation. (b) Outer membrane is unusually rich in proteins (23), and is even claimed to be somewhat rigid (38) presumably as a result of protein-to-protein interactions. The rigidity, high degree of protein-to-protein interaction, and the possible presence of proteins at unsealed edges of membrane fragments would all interfere with the resealing process, as well as the redistribution of various components into their proper positions. These factors would then produce vesicles which are incompletely resealed, or have components still aligned incorrectly, or both.

Two lines of evidence support the hypothesis described above. (a) Outer membrane vesicles from the Re mutant were less leaky than vesicles from the other strains (Fig. 3). This is understandable in view of the fact that Re mutants have fewer anionic groups in their lipopolysaccharides (22) and have much lower protein content in their outer membrane (21), in comparison with Rc and wild type strains. (b) Three conditions are needed in order to minimize the penetration of larger saccharides into vesicles. Among the conditions trypsin treatment would degrade proteins and might make the membrane more flexible, Mg²⁺ would neutralize the negative charges of lipopolysaccharide and reduce electrostatic repulsion, and heating-slow cooling cycle would help the membrane components to settle into the proper arrangement. It seems most likely, therefore, that these treatments reseal the “cracks” in damaged membrane vesicles.

This study showed that the outer membrane allows the penetration of di- and trisaccharides. Since phospholipid bilayers are essentially impermeable for sugars larger than pentoses (40), we have to assume that components other than phospholipids are responsible for this high permeability. Since lipopolysaccharide is a unique component of the outer membrane, it was thought to be a good candidate for such a component. However, mixed bilayers containing both lipopolysaccharide and phospholipids had permeability properties very similar to those of phospholipid bilayers (1). We thus have to conclude that the protein (or proteins) in the outer membrane must be responsible for its high permeability. Our observation that the partial removal of lipopolysaccharide did not change the saccharide permeability of cells (see “Results”) is also consistent with this conclusion. Recently Inouye (41) has proposed, from theoretical considerations, that the Braun lipoprotein (37, 38) might aggregate and form hydrophilic channels that penetrate through the thickness of the outer membrane. We have succeeded in reconstituting sucrose-permeable outer membrane vesicles from outer membrane
proteins, lipopolysaccharides, and phospholipids (42); thus it may become possible to identify the permeability-conferring protein with this technique.

The data presented here do not distinguish between the penetration through pores and the diffusion mediated by carrier proteins. However, evidence to be presented elsewhere appears to support the pore mechanism.

Finally, we must emphasize that the hydrophilic pores are not the only pathway through which small molecules move across the outer membrane. Firstly, there are hydrophilic molecules that are too large to go through the pore, yet must diffuse through the outer membrane to support the growth of cells. It is most interesting that various phage and colicin receptor proteins (carriers?) are obviously needed for the transmembranous diffusion of these large molecules. Secondly, the alteration of lipopolysaccharide structure drastically alters the permeability of the outer membrane to certain dyes and antibiotics (7-13), which may become possible to identify the permeability-conferring protein with this technique. However, evidence to be presented elsewhere appears to support the pore mechanism.

Examples include the binding of vitamin B_{12} (1357 daltons) by colicin B-, E-receptor (44), that of ferrichrome (740 daltons) by T5-, @O-, colicin M-receptor (45). In these cases the "receptor proteins" on the surface of E. coli and S. typhimurium were found to bind these large molecules specifically. Examples include the binding of vitamin B_{12} (1357 daltons) by colicin E-receptor (43), that of enterocin (746 daltons) by colicin B-, I-, V-receptor (44). and that of ferrichrome (740 daltons) by T5-, @O-, colicin M-receptor (45). In these cases the binding by specific proteins is obviously needed for the transmembranous diffusion of these large molecules. Secondly, the alteration of lipopolysaccharide structure drastically alters the permeability of the outer membrane to certain dyes and antibiotics (7-13), which therefore must diffuse through the outer membrane by a different mechanism. We believe that these substances move across the membrane by first dissolving into the hydrophobic interior of the membrane; the properties of this "hydrophobic pathway" will be discussed in a separate paper.  

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T Nakae and H Nikaido

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