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Partitioning of hydrophobic probes into lipopolysaccharide bilayers

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Lipophilic solutes permeate rapidly through lipid bilayer membranes. However, the outer membrane of enteric bacteria, which is composed of a lipopolysaccharide monolayer outer leaflet and the glycerophospholipid inner leaflet, shows extremely low permeability to hydrophobic solutes. In order to examine the cause of this exceptionally low permeability, the lipid/water partition behavior of various lipophilic probes was determined by using lipopolysaccharides of various chemotypes and glycerophospholipids. With all probes, under many different conditions, the lipopolysaccharide/water partition coefficients were generally about an order of magnitude smaller than the phospholipid/water partition coefficients, and this result is consistent with the low permeability of the lipopolysaccharide monolayer, and hence the asymmetric bilayer found in the outer membrane. Furthermore, organic polycations significantly increased the partition of *N*-phenylnaphthylamine into lipopolysaccharides, a result again consistent with the permeability-increasing effect of such cations on intact outer membrane. Very defective, 'deep rough' lipopolysaccharides of chemotypes Rd_2, Rd_1 and Re, had only slightly (20–75%) higher partition coefficients in comparison with the more complete lipopolysaccharides, and this difference is probably not enough to explain the approximately 100-fold increase in lipophile permeability seen in deep rough strains.

Introduction

Small, hydrophobic molecules penetrate rapidly across lipid bilayers and biological membranes containing such bilayers [1]. The lipid bilayer found in the outer membrane of enteric bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, constitutes a striking exception in that it shows very low permeability toward hydrophobic solutes [2]. The outer membrane bilayer is also unusual in its structure, its outer leaflet being made entirely of LPS molecules rather than glycerophospholipids [3]. Since LPS has an unusual structure in which six or seven fatty acid chains are linked to a common backbone composed of a glucosamine disaccharide [4], the simplest hypothesis is to assume that the LPS monolayer constitutes the barrier for the diffusion of lipophilic solutes.

Interestingly, the outer membrane permeability to lipophilic compounds increases dramatically in 'deep rough' mutants that produce LPS with very short saccharide chains [2]. Although this result seems to suggest, at first sight, that the saccharide chains of LPS function as a permeability barrier, the situation is more complex as these mutants cannot incorporate normal levels of proteins into the outer membrane, and presumably as a result contain domains of glycerophospholipid bilayers in the outer membrane [3]. Thus it seems plausible that the increased permeability is due to the flux of solutes through these glycerophospholipid bilayer domains, rather than through the monolayers of altered LPS [5]. Finally, treatment of the cells of most enteric bacteria with polycations such as oligolysine and polymyxin nonapeptide, which bind to the polyanionic LPS, disorganizes the outer membrane architecture [6] and at the same time drastically increases its permeability to lipophiles [7].

All these observations suggest that the low permeability to lipophiles may be due to the difficulty they have in partitioning into the hydrophobic interior of

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Abbreviations: LPS, lipopolysaccharide; NPN, *N*-phenylnaphthylamine; DTBN, di-*t*-butylnitroxide.

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LPS monolayer. However, this point has never been established experimentally. Furthermore, an experimental study is essential in excluding alternative hypotheses, for example that attributing the barrier function mainly to the saccharide layer, rather than to the hydrophobic interior, of the LPS monolayer. The ideal model system for the study of outer membrane permeability would be an asymmetric bilayer, one side exclusively containing LPS, and the other composed of glycerophospholipids. Indeed very recently a planar bilayer of this type has been made successfully [8]. However, it is difficult to measure permeability of uncharged solutes with such bilayers, because of the instability of the bilayer and the problems associated with the presence of the unstirred layer next to the membrane. Another approach is to test the permeability of closed vesicles made of symmetric bilayers of LPS. Temperature-induced increase in fluorescence of LPS dispersions made in the presence of carboxyfluorescein suggested that such closed vesicles or liposomes with this fluorescent dye entrapped within were produced from pure LPS preparations [9]. However, we have not been able to prepare vesicles of this type [10] (Vaara, M. and Nikaido, H., unpublished results), assayed on the basis of entrapment of radioactive hydrophilic solutes, such as [^{14}C]sucrose or lactose, even though we used conditions thought to favor the stabilization of LPS bilayer, such as high temperature (up to 80°C), addition of divalent cations, and use of very defective Re-type [5] LPS. Because of these results, we chose to examine the partition of hydrophobic solutes between the aqueous phase and the hydrocarbon interior of LPS bilayer. Since the diffusion rate across lipid bilayers depends largely on the magnitude of partition coefficients between water and the bilayer interior [1], measurement of this type is expected to shed light on the permeability of LPS monolayers. Although pure LPS does not easily produce closed vesicles, bilayers of limited size are readily produced when LPS is resuspended in aqueous media [11], and they could be used for measurements of this type. The results confirm at least qualitatively that lipophiles have difficulty in dissolving into the hydrocarbon region of LPS.

Materials and Methods

LPS

LPS was isolated from rough mutants of *Salmonella*, shown in Table I. Bacteria were grown overnight at 37°C on glucose-enriched nutrient agar [18], and LPS was isolated by the phenol-chloroform-petroleum ether extraction method [19]. In most experiments, LPS preparations were used in this 'natural' form, i.e., as mixed salts. In some experiments, sodium salt of the LPS from SH9178, prepared by electrodialysis [20], was used.

TABLE I

The LPS material used in this study

Chemotype	Strain	Species	No. of different batches studied	Reference
Ra	SH9013	*S. typhimurium*	1	*his-515* in 12
Rb_2	SH1909	*S. typhimurium*	1	13
Rb_2	SH5014	*S. typhimurium*	3	14, 15
Rb_2	SH6482	*S. typhimurium*	5	15
Rb_2	SH9178	*S. typhimurium*	3	15
Rd_1	SL1032	*S. typhimurium*	2	16
Rd_1	SH7639	*S. typhimurium*	1	17
Rd_1	SH7764	*S. typhimurium*	1	M. Vaara, unpubl.
Rd_1	SH7766	*S. typhimurium*	1	M. Vaara, unpubl.
Rd_1	SH7768	*S. typhimurium*	1	M. Vaara, unpubl.
Rd_1	SH7774	*S. typhimurium*	1	M. Vaara, unpubl.
Rd_2	SL1181	*S. typhimurium*	1	18
Re	SL0320	*S. minnesota*	1	19

For determination of partition coefficients, fresh suspension of LPS were made in appropriate assay buffers specified. Usually, clear, homogeneous dispersions were obtained. However, some preparations gave slightly opalescent, but still homogeneous, dispersions.

In most partition studies, the same amount (defined by weight) of various LPS preparations was used. In the NPN partition studies, however, the amount of lipidic material used was defined on a molar basis. Following values of molecular weight (calculated on the basis of structure [5]) were used for the different LPS chemotypes: Ra, 4000; Rb_2, 3600; Rd_1, 2900; Rd_2, 2500; and Re, 2300. Finally, the volume of the LPS phase was calculated by assuming that the density of rough LPS was approx. 1.4 g/cm^3 [21].

Phospholipids

Phospholipids were extracted from *S. typhimurium* by the method of Folch et al. [22], washed once with the theoretical upper phase mixture, dried, dissolved in chloroform/methanol (2 : 1, v/v), and stored at −70°C. Phospholipid dispersions (liposomes) were prepared by drying the lipids as a thin film at the bottom of a test tube, resuspending them in distilled water, and sonicating them with a probe-type sonicator until the suspension became clear. This suspension was then diluted with an appropriate buffer. Phosphatidylcholine-dicetyl phosphate liposomes were made using acetone-washed egg phosphatidylcholine and dicetyl phosphate (molar ratio 31 : 1) as described earlier [23], except that the dried lipid film was suspended in 0.02 M Hepes-NaOH buffer (pH 7.2). In the calculations of partition coefficients, the volume of the lipid phase was calculated on the basis of a density of 1.0 g/cm^3.

Partitioning of an electron spin resonance (ESR) probe

The spin-label probe was DTBN enriched in ^{12}C and ^{2}H, synthesized as described recently [24]. The probe will be called pd-^{12}C-DTBN in this paper. 5 μl of a 2 mM solution of this probe was added to the lipid samples (5 mg in 100 μl of water) in a Teflon capillary tube [25]. The tube was then sealed, deaerated, and allowed to equilibrate at the set temperature in a Varian E-12 ESR spectrometer, equipped with a variable temperature attachment and Varian E-900 data system. The spectra were obtained with a scan range of 50 G, scanning time of 8 min, modulation amplitude of 0.04 G, and the receiver gain of 1,250. The weak signals from the probe in the hydrophobic phase were detected, when necessary, by increasing the sensitivity 10-fold, by using a modulation amplitude of 0.125 G and a receiver gain of 4000.

The probe in the lipid phase produced a signal displaced downfield from the larger signal arising from the probe in the aqueous phase. The intensities of these signals were measured in the high field peak (where the separation was maximal) from the areas of the positive peaks [26]. The water/lipid partition coefficient (P) was calculated as follows.

$$P = \frac{\text{(intensity of hydrophobic signal/volume of lipid phase)}}{\text{(intensity of aqueous signal/volume of aqueous phase)}}$$

Partition coefficient of NPN

The stock solution of NPN (obtained from Sigma; 1 mM in methanol), made immediately before use, was diluted tenfold in 0.02 M Hepes-NaOH buffer (pH 7.2). 20 μl of this solution, containing 2 nmol of NPN, were added to lipid samples (20 nmol of LPS or 70 nmol of phospholipids in 2 ml of Hepes-NaOH buffer, unless stated otherwise). After 15 min at room temperature, the fluorescence was measured with a Perkin-Elmer MPF-44B spectrofluorometer (excitation at 360 nm; emission at 425 nm; slit width 6 nm). Both the lipid background fluorescence (with lipid samples without NPN) and the fluorescence due to NPN in the aqueous phase (measured without adding any lipid) were subtracted from the observed reading, to yield the corrected fluorescence, I. In order to get the maximal intensity of fluorescence, a large excess of Triton X-100 (1 mg/ml) was used, and this yielded the maximal fluorescence, I_M. The partition coefficient, P, was calculated as follows:

$$P = (I/\text{volume of lipid phase})/((I_M - I)/\text{volume of aqueous phase})$$

Partitioning of [^{14}C]indole

Radioactive indole (4 nmol; specific activity 52 mCi/mmol; obtained from Research Products International) in 10 μl of 0.02 M Hepes-NaOH buffer (pH 7.2) was added to the lipid suspension (1 mg of LPS or phospholipids in 90 μl of Hepes buffer). After 15 min at room temperature, the suspension was centrifuged at 105 000 × g for 10 min in a Beckman airfuge, a procedure that resulted in a complete pelleting of LPS and phospholipids. The radioactivity in 50 μl of the supernatant was measured, and the total radioactivity in the aqueous phase was obtained by multiplying this value by 2. The radioactivity in the lipid phase was then obtained by subtracting the aqueous phase radioactivity from the total radioactivity added to the system. The partition coefficient, P, was calculated as follows.

$$P = \frac{\text{(radioactivity in lipids/volume of lipid phase)}}{\text{(radioactivity in aqueous phase/volume of the aqueous phase)}}$$

Separate control experiments showed that there was no significant adsorption of the label onto the polyallomer centrifuge tubes and polypropylene pipette tips.

When the amount of the probe was varied from 4 to 24 and finally to 104 nmol, the partition coefficients obtained with the phosphatidylcholine-dicetyl phosphate liposomes were 85.4, 85.2, and 76.4, respectively, showing that the values of the coefficients were not affected significantly. Furthermore, doubling the amount of phospholipids did not change the value of the partition coefficient obtained.

Partitioning of [^{14}C]phenol

Radioactive phenol in 10 μl of water (50 nmol, obtained from ICN radiochemicals and diluted with non-radioactive phenol to the specific activity of 2 mCi/mmol) was added to lipid suspensions (5 mg of LPS or phospholipids in water). After 15 min at room temperature, the suspensions were centrifuged and the partition coefficient was determined as described above for [^{14}C]indole.

Results

Partitioning of ESR probe

LPS/water partition coefficients of pd-^{12}C-DTBN were determined at 40 and 49°C (Fig. 1). We used this probe rather than the more commonly used TEMPO, because the probe signals are narrower, and lack the ^{13}C satellite peaks found with TEMPO. Thus the integration of the peak areas can be done more accurately with this probe. As seen in Table II, at 40°C the LPS/water partition coefficients of pd-^{12}C-DTBN were approximately one-tenth of the phospholipid/water partition coefficients. Increasing the temperature to 49°C generally resulted in an approx. 2-fold increase in the partition coefficients. Finally, although the partition was slightly higher into a deep rough, Rd_2 type LPS than into the standard rough LPS, the difference was very small (about 20% at 40°C).

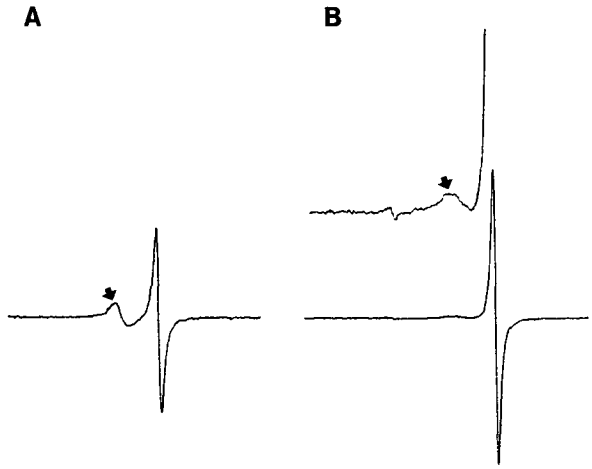

Fig. 1. ESR spectra (original trace) of pd-^{12}C-DTBN in aqueous dispersions of *S. typhimurium* phospholipids (A) and LPS (B). Only the high field peaks are shown. The low field part of the LPS spectra was also recorded with a 10-fold higher sensitivity as described in Methods, in order to quantitate more accurately the signal of pd-^{12}C-DTBN in the lipid environment (arrow).

Partitioning of NPN

The behavior of an uncharged, fluorescent probe, NPN, was investigated under various conditions (Table III). NPN shows strong fluorescence with the emission maximum at 425 nm essentially only when it is in a hydrophobic environment, and thus is a very suitable probe for partition studies. As seen, the partition coefficient into LPS was 25% and 17% of that into phospholipids, in the absence and presence, respectively, of 1 mM Ca^{2+}. Again, more of the probe partitioned into deep rough LPS than into the standard rough LPS, but the partition coefficients into deep rough LPS still remained much lower than those into phospholipids. Interestingly, the partition coefficient into LPS became significantly lower when the LPS was converted into a sodium salt; in the presence of Ca^{2+}, the difference between phospholipids and LPS was about 8-fold.

TABLE II

Lipid/water partition coefficients of pd-^{12}C-DTBN

For details of procedure, see Materials and Methods. The ratios of volumes of aqueous phase to the lipid phase were 19.9, 28, and 33.3 in experiments using phospholipids, Rb_2 LPS, and Rd_2 LPS, respectively.

Material	Temperature (°C)	Hydrophobic signal/ aqueous signal	Partition coefficient
S. typhimurium phospholipids	40	0.17	3.4
	49	0.28	5.5
LPS (Rb_2)	40	0.014	0.39
	49	0.031	0.87
LPS (Rd_2)	40	0.015	0.50
	49	0.038	1.27

TABLE III

Lipid/water partition coefficients of NPN measured by spectrofluorometry

For procedures, see Materials and Methods. The number of experiments performed is indicated within parentheses.

Material	No. of batches studied	Partition coefficient	
		in the absence of Ca^{2+}	in the presence of 1 mM Ca^{2+}
S. typhimurium phospholipids	2	16389±1704 (5)	16388± 608 (3)
LPS (Ra and Rb_2)	13	4090±1459 (31)	2719± 856 (16)
LPS (Rd_1, Rd_2, Re)	9	7135±2329 (17)	5527±2270 (4)
LPS (Rb_2, Na salt)	1	2501± 602 (3)	2113± 111 (2)

Because the partitioning of NPN was easy to determine experimentally, we have examined the effect of various inorganic and organic polycations in this process. When an Rb_2 LPS from SH6482 was used, 0.1 mM Ca^{2+} significantly decreased the partition coefficient of NPN from 5900 (in the control without any added cation) to 1740. Mg^{2+} (also at 0.1 mM) tended to increase the partition coefficient slightly, in this experiment to 7740. Other divalent and trivalent cations (Al^{3+}, Fe^{3+}, Ba^{2+}, Zn^{2+}, Pb^{2+}, and Cu^{2+}), all tested at 0.1 mM, decreased partition of NPN significantly. Among them, Cu^{2+} consistently had the strongest effect, in this experiment decreasing the NPN partition coefficient to only 170. In another experiment using the same LPS and organic polycations, 16 μM spermidine and putrescine increased the NPN partition coefficients from 2600 (without any added cation) to 6970 and 5140, respectively. Finally, the polycationic compound polymyxin nonapeptide [6,7] was able to reverse the powerful effect of Cu^{2+}. With an Rb_2 LPS, the addition of 0.1 mM Cu^{2+} decreased the NPN partition coefficient from 6130 to 360; however, if 20 μM polymyxin nonapeptide was added together with Cu^{2+}, the partition coefficient showed hardly any decrease, only to 4200.

TABLE IV

Lipid/water partition coefficients of [^{14}C]indole

When more than two experiments were performed, mean value± standard deviation is shown, with the number of experiments in parenthesis. n.d., not determined.

Material	No. of batches studied	Partition coefficient	
		in the absence of Ca^{2+}	in the presence of 30 mM Ca^{2+}
S. typhimurium phospholipids	2	128± 6 (3)	140
Phosphatidylcholine-dicetyl phosphate	2	94±11 (3)	103
LPS (Ra and Rb_2)	5	38± 5 (5)	30±4 (5)
LPS (Rb_2, Na salt)	1	30	n.d.

TABLE V

Lipid/water partition coefficients of [^{14}C]phenol

When more than two experiments were performed, the mean ± standard deviation is shown with the number of experiments in parenthesis. n.d., not determined.

Material	No. of batches studied	Partition coefficient	
		in the absence of Ca^{2+}	in the presence 30 mM Ca^{2+}
S. typhimurium phospholipids	1	9.6	N.D.
LPS (Rb_2)	2	5.9 ± 0.4 (3)	1.4
LPS (Rd_1, Rd_2)	3	7.5 ± 1.9 (5)	0.7

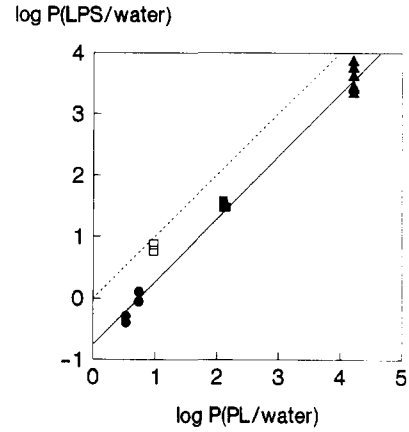

Fig. 2. Correlation between LPS/water partition coefficients and phospholipid/water partition coefficients. All the data from Tables II through V, except those measured with egg phosphatidylcholine-dicetyl phosphate system, are shown in this figure. The broken line shows the expected correlation if the LPS/water partition coefficients were equal to phospholipid/water partition coefficients. It is seen that the observed LPS/water partition coefficients are usually nearly an order of magnitude lower than the phospholipid/water partition coefficients, in spite of the wide variety of LPS species, conditions (temperature, presence or absence of Ca^{2+}), and the probes used in this study. Symbols: ●, pd-^{12}C-DTBN; □, phenol (in the absence of Ca^{2+}); ■, indole; and ▲ NPN.

Partition behavior of [^{14}C]indole and [^{14}C]phenol

A behavior similar to NPN was seen when the partitioning of [^{14}C]indole and [^{14}C]phenol was measured by assaying for radioactivity (Tables IV and V). With the latter compound, the addition of Ca^{2+} had an especially strong effect in decreasing the partition coefficient in the LPS/buffer system; this point will be examined in Discussion. The partition coefficients of these compounds in phospholipid/water system (128 and 9.6) were fairly close to their published 1-octanol/water partition coefficients (135 and 31 [27]).

Discussion

We have shown in this study that neutral, lipophilic molecules partition much less effectively into the lipid interior of LPS than into that of phospholipids. This was true with probes covering a very wide range of hydrophobicity under a variety of conditions (Fig. 2). Since the partitioning of hydrophobic solutes into lipid interior increases with increasing fluidity of the lipid domain [1], the simplest interpretation of these results is that they are caused by the lower fluidity of the LPS hydrocarbons. Indeed, the hydrocarbon interior of LPS dispersions was shown to have very low fluidity, as deduced from the maximum hyperfine splitting values of an ESR probe [28] and from the sharpness of reflections caused by spacing between hydrocarbon chains in X-ray scattering studies [29]. Such low fluidity is also expected from the structure of LPS, containing 6–7 fatty acid chains linked to the common backbone, and containing elaborate oligo- or polysaccharide head groups. The effects of structural changes of lipids in these directions can be seen in the major lipid of *Thermus*, a thermophilic bacterium. This lipid contains three fatty acids linked to the common backbone rather than two fatty acids found in the usual glycerophospholipids, and its head group consists of a tetrasaccharide; these modifications apparently make the lipid interior not excessively fluid (and thus not allowing excessive leakage) even at 90°C, the growth temperature of this organism [30,31].

Although it has been claimed that the hydrocarbon chains of LPS undergoes a thermal transition at fairly low temperatures in the range of 22–40°C [9,32–34], the presence of transition does not immediately indicate that the fluidity above the transition point is very high, and thus our data are not necessarily at conflict with these published results. However, the ratio of LPS/water partition coefficients relative to the phospholipid/water partition coefficients was quite similar regardless of whether the assay was carried out at 40 and 49°C as in the ESR experiments or at room temperature (around 25°C) in other experiments, and we obtained no suggestion of large-scale changes of LPS fluidity in the range mentioned in these publications. We also note that cooperative thermal transition in this temperature range was not detected by an earlier ESR experiment by one of us [28], nor by microcalorimetry using intact *E. coli* cells [35,36], and we believe that the reported transitions are artefacts perhaps caused by the imperfect packing of LPS molecules [37]. Thus, the LPS molecules, once dissociated during the course of preparation, sometimes may not be packed tightly, possibly because it is difficult to stack the complex saccharide chains correctly against each other.

Our observations explain, at least qualitatively, why the lipid bilayer of the outer membrane constitutes a barrier of unusually low permeability. Thus attachment of more than two hydrocarbon chains to a common

backbone structure inhibits the creation of disordered structure by decreasing the movement of one molecule against another. Large saccharide head groups should further stabilize the monolayer structure by producing extensive network of intermolecular hydrogen bonds, as has been postulated for other types of glycolipids [38,39]. Indeed, intermolecular association between the saccharide chains of LPS was experimentally demonstrated [40].

However, our observation by itself does not provide a complete quantitative explanation of the barrier function of the outer membrane. Thus a hydrophobic penicillin, nafcillin, was shown to penetrate through the intact outer membrane of *S. typhimurium* at a rate at least 100-fold lower than its rate of penetration through the cytoplasmic membrane [2]. Since the permeability coefficients of various solutes of about the same size are proportional to their partition coefficients [1], on this basis we could have expected LPS/water partition coefficients that were 100-fold lower than phospholipid/water partition coefficients. As shown in Tables II–V, the difference observed was at best only 10-fold. One likely explanation for this apparent discrepancy is the following. The proportionality relationship mentioned above holds only for a set of solutes diffusing across a given bilayer membrane. Here we are comparing the diffusion rates of a given solute across bilayers of very different fluidity, and clearly the rates of solute diffusion within the viscous hydrocarbon layer should also affect the penetration rate, in addition to the partition coefficient of the solute [1]. Theory predicts that the permeability coefficient is proportional to the product of the partition coefficient and the intramembranous diffusion coefficient [1,41]. Thus if the lower fluidity of the LPS hydrocarbon domain decreases the diffusion coefficient also by about an order of magnitude, the combined result would be a 100-fold lower penetration rates.

There is another partial explanation for the discrepancy. As is well known, various parts of the membrane interior have greatly different values of fluidity [42]. Yet experiments of the type carried out here only measures the partition of solutes into the most accommodating region of the hydrocarbon interior [41]. Thus the partition coefficients obtained in this manner cannot quantitatively predict the permeation rates [41], which are affected most strongly by the partitioning into the least accommodating region of the bilayer. Additionally, since LPS makes bilayers of only limited sizes [11], many lipophile molecules may partition into the edges of such structures, where the bilayer must be highly distorted. This will also result in the overestimation of the LPS/water partition coefficients.

Some of our own data indeed suggest the heterogeneity within the hydrophobic interior of the LPS bilayer. It is known that small and not very hydrophobic solutes tend to be partitioned into a less hydrophobic domain close to the head group, and this is suggested by a phospholipid/water partition coefficient which is significantly lower than the octanol/water coefficient [41]. Among the solutes we tested, a small, rather hydrophilic probe, phenol, indeed behaved in this way, exhibiting a phospholipid/water partition coefficient three times lower than in the octanol/water system (see Results). In the LPS bilayer, phenol also appeared to partition in the region of the hydrocarbon close to the complex head group, because the addition of Ca^{2+} decreased dramatically the partitioning of phenol (Table V), presumably by tightening the lateral interaction between the head groups by decreasing electrostatic repulsion between the anionic groups, an effect that should increase the order of the hydrocarbon domain located adjacent to the head groups. In contrast, large or very hydrophobic solute will partition predominantly into the more fluid, central portion of the bilayer; indeed this is suggested by the observation that the more hydrophobic indole had a higher partition coefficient in phospholipid/water system, in comparison with the octanol/water system (see Results). As predicted, the LPS/water partition coefficients of indole and another hydrophobic probe, NPN, were little affected by Ca^{2+} that acts on the head group (Tables III and IV).

When bacteria produce very defective 'deep rough' LPS, containing only 3-deoxyoctulosonic acid or that plus heptose residues in the saccharide chain (chemotypes Rd_1, Rd_2, and Re), their outer membrane becomes much more permeable to lipophilic solutes [2]. A priori, this could be due to the higher permeability of the monolayer composed of such altered LPS molecules. Alternatively, because deep rough mutants were found to contain increased amounts of glycerophospholipids in the outer membrane, and because mutants that produce outer membranes containing normal LPS but increased amounts of phospholipids show similar increased permeability, we favored the hypothesis that lipophilic solutes cross the outer membrane mainly via glycerophospholipid bilayer regions. In this connection, it was of interest to compare the partition coefficients into deep rough LPS and normal (rough) LPS bilayers. Such comparisons (Tables II, III, and V) showed that deep rough LPS tended to produce higher values of partition coefficients; this is in agreement, qualitatively, with the observation of Brandenburg and Seydel [9], who concluded from the hexatriene fluorescence polarization data that the lipid interior is more fluid in deep rough LPS. However, unlike the order parameter obtained by Brandenburg and Seydel [9], the partition coefficients obtained by us are somewhat more directly related to the lipophile permeation rates. Even when allowances are made for possible increases in intramembrane diffusion rates (see above), the observed difference (1.2–1.8-fold) was so small that it seems

rather unlikely to explain the 100-fold difference in the permeation rates observed [2]. These results thus favor the hypothesis that the presence of glycerophospholipid bilayers, rather than the alteration of the structure of LPS, plays a more important role in the increase of outer membrane permeability in deep rough mutants.

Finally, a preliminary study of several inorganic and organic multivalent cations showed that organic polycations increased the partition of NPN into the LPS bilayer. We have not examined the experimental parameters of this system in detail, and indeed NPN, which presumably partitions mostly in the central, most fluid part of the bilayer (see above) could have been an inappropriate probe for examining the structure of the region of bilayer adjacent to the head group. Nevertheless, the observed increase in NPN partition suggests that increased outer membrane permeability caused by the addition of organic polycations to intact cells [6,7] may ultimately be explained by the alteration of the physical structure of bilayer, and encourages further experiments in this direction.

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