Professor W. Hengstenberg and Dr. U. Schmidt-Aderjan of M.P.I. for their cooperation with the nitration of tyrosine.

References

Akasaka, K. (1978) in *Proceedings of the European Conference on NMR of Macromolecules* (Conti, F., Ed.) pp 475–483, Lerici, Rome.

Campbell, I. D., Dobson, C. M., Williams, R. J. P., & Xavier, A. V. (1973) *J. Magn. Reson. 11*, 172–181.

Carrington, A., & McLachlan, A. D. (1967) in *Introduction to Magnetic Resonance*, Chapter 12, Harper and Row, New York.

Fujii, S., Akasaka, K., & Hatano, H. (1980) *J. Biochem. (Tokyo) 88*, 789–796.

Greenstein, J. P., & Winitz, M. (1961) in *Chemistry of the Amino Acid*, Vol. 1, pp 492–494, Wiley, New York.

Hayashi, K., Shimoda, T., Imoto, T., & Funatsu, M. (1968) *J. Biochem. (Tokyo) 64*, 365–370.

Hirono, S., Nakamura, K. T., Iitaka, Y., & Mitsui, Y. (1979) *J. Mol. Biol. 131*, 855–869.

Huber, R., Kukla, D., Rühlmann, A., & Steigemann, W. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 141–151.

Ikenaka, T., Odani, S., Sakai, M., Nabeshima, Y., Sato, S., & Murao, S. (1974) *J. Biochem. (Tokyo) 76*, 1191–1210.

Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., & Rupley, J. A. (1972) *Enzymes, 3rd Ed. 7*, 665–686.

Inouye, K., Tonomura, B., Hiromi, K., Sato, S., & Murao, S. (1977) *J. Biochem. (Tokyo) 82*, 1207–1215.

Lee, B., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 379–400.

Mitsui, Y., Satow, Y., Sakamaki, T., & Iitaka, Y. (1977) *J. Biochem. (Tokyo) 82*, 295–298.

Mitsui, Y., Satow, Y., Watanabe, Y., & Iitaka, Y. (1979a) *J. Mol. Biol. 131*, 697–724.

Mitsui, Y., Satow, Y., Watanabe, Y., Hirono, S., & Iitaka, Y. (1979b) *Nature (London) 277*, 447–452.

Murao, S., & Sato, S. (1972) *Agric. Biol. Chem. 36*, 160–163.

Perkins, S. J., & Dwek, R. A. (1980) *Biochemistry 19*, 245–258.

Sato, S., & Murao, S. (1973) *Agric. Biol. Chem. 37*, 1067–1074.

Satow, Y., Watanabe, Y., & Mitsui, Y. (1980) *J. Biochem. (Tokyo) 88*, 1739–1755.

Snyder, G. H., Rowan, R. R., III, & Sykes, B. D. (1976) *Biochemistry 15*, 2275–2283.

Tojo, T., Hamaguchi, K., Imanishi, M., & Amano, T. (1966) *J. Biochem. (Tokyo) 60*, 538–542.

Wüthrich, K., & Wagner, G. (1975) *FEBS Lett. 50*, 265–268.

Wüthrich, K., Wagner, G., Richarz, R., & De Marco, A. (1977) in *NMR in Biology* (Dwek, R. A., Campbell, I. D., Richards, R. E., & Williams, R. J. P., Eds.) pp 51–62, Academic Press, New York.

Persistence of Segregated Phospholipid Domains in Phospholipid–Lipopolysaccharide Mixed Bilayers: Studies with Spin-Labeled Phospholipids†

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ABSTRACT: When lipopolysaccharides from *Escherichia coli* B were sonicated together with pure spin-labeled phospholipids without the addition of unlabeled phospholipids, extensive line broadening was observed due to the close proximity of spin-labeled molecules to each other, a result suggesting that spin-labeled phospholipids existed in segregated domains containing few lipopolysaccharide molecules. Such mixed bilayers were incubated under various conditions, including the addition of NaCl and $MgCl_2$ to the medium and the incorporation of the major outer membrane protein, porin, into the bilayer, and the intermixing of the domains was followed by the decrease in line width. The diffusion of the labeled phospholipids into lipopolysaccharide domains was hardly detectable when the mixed bilayer contained spin-labeled phospholipids and lipopolysaccharide in approximately equimolar ratios. Although progressive diffusion was observed when a 17-fold molar excess of lipopolysaccharide was present, it was very slow even under the optimal conditions, usually requiring several days for a nearly complete mixing. In another series of experiments, spin-labeled phospholipids were diluted with a 100-fold excess of unlabeled phospholipids and then mixed with lipopolysaccharides. In these experiments, the fluidity of the domains containing spin-labeled phospholipids was shown to be identical, even after 3 days of incubation, with the fluidity of bilayers containing only phospholipids, in contrast to the expectation of the diminished fluidity if phospholipid molecules became finely interspersed with lipopolysaccharide molecules. These two different lines of approach therefore supported the idea that phospholipid (and most probably lipopolysaccharide) domains in mixed bilayers tend to be rather stable and persist for long periods of time.

The outer membrane of Gram-negative bacteria contains two classes of amphiphilic lipids, phospholipids and lipopolysaccharides [for a review, see Nikaido & Nakae (1979)]. In *Escherichia coli* and *Salmonella typhimurium*, about 80% of the phospholipids are phosphatidylethanolamine (PE),[1] and

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[1] Abbreviations used: LPS, lipopolysaccharide; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PE*, spin-labeled PE; PG*, spin-labeled PG; 5- (or 12-) nitroxide stearate, 4,4-dimethyloxazolidinyl-*N*-oxy derivative of 5- (or 12-) ketostearic acid; ESR, electron spin resonance; Hepes, *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

0006-2960/81/0420-0523$01.00/0

about 17% corresponds to phosphatidylglycerol (PG) (Osborn et al., 1972). Lipopolysaccharide (LPS) of these genera contains, in a single molecule, six fatty acid residues, all linked to a backbone of glucosaminylglucosamine phosphate, and also large numbers of negatively charged groups in the most proximal part of its polysaccharide portion (Nikaido & Nakae, 1979).

LPS is synthesized on the cytoplasmic membrane, and the pioneering work of Rothfield and co-workers showed that biosynthetic intermediates of LPS must exist in a form associated with phospholipids in order for the biosynthetic reactions to continue (Rothfield & Romeo, 1971). Furthermore, they showed that LPS added to the aqueous subphase of a phospholipid monolayer became inserted into the monolayer (Romeo et al., 1970) and that addition of LPS to bacterial PE in an aqueous environment produced liposomes composed of mixed LPS–PE bilayers (Weiser & Rothfield, 1968). These studies were thought to suggest the presence of strong phospholipid-to-LPS interactions. Furthermore, among phospholipids, PE appeared to have an especially strong affinity to LPS. Thus, only PE produced a highly homogeneous phospholipid–LPS complex in the liposome system (Weiser & Rothfield, 1968), the insertion of LPS into phospholipid monolayer was especially efficient with the monolayer of PE (Romeo et al., 1970), and more recently Fried & Rothfield (1978) concluded, from the study of the pressure–area relationship of monolayers, that 16 molecules of PE and one molecule of LPS formed a tight, stoichiometric complex.

It is expected that during its biosynthesis on the cytoplasmic membrane LPS would be surrounded by the phospholipid molecules. Thus, the presumed strong interaction between LPS and phospholipid will be advantageous in this situation. However, recent results suggest that such a strong interaction might act as a deterrent in another situation. Thus, studies in our laboratory have shown that the synthesized LPS, in its final location, i.e., the outer membrane, occupied domains which were completely segregated from those occupied by phospholipids (Nikaido et al., 1977). In fact, studies involving enzyme digestion, covalent labeling with impermeant reagents, and freeze–fracture electron microscopy suggested strongly that LPS was exclusively localized in the outer half of the outer membrane whereas the location of phospholipids was limited essentially to the inner half (Smit et al., 1975; Kamio & Nikaido, 1976; Funahara & Nikaido, 1980). If strong affinity did exist between LPS and phospholipids, it could be expected to hinder the formation of such segregated domains and to destabilize them after their formation.

These considerations prompted us to reexamine the interactions between phospholipid and LPS molecules by utilizing spin-labeled PE (PE*) and spin-labeled PG (PG*) as reporter molecules. The results suggest that in bilayers both phospholipid and LPS molecules have strong tendencies to remain in their own, homogeneous domains and that two-dimensional mixing of these domains is an extremely slow process, especially when PE is the phospholipid component.

Materials and Methods

Spin-Labeled Compounds. 12-Nitroxide stearate was obtained from Syva Associates (Palo Alto, CA). Spin-labeled phosphatidylcholine was synthesized from egg yolk lecithin by the method of Hubbell & McConnell (1971) and was then converted to PE* and PG* by head-group exchange reaction catalyzed by phospholipase D (Takeuchi et al., 1978). PE* and PG* therefore retained, at the *sn*-1 position, the fatty acid residues originally present in egg yolk lecithin and contained, at the *sn*-2 position, 12-nitroxide stearate residues.

Bacterial Growth and Preparations of LPS, Phospholipids, and Porin. E. coli B was grown in L broth (Bertani, 1951) (glucose omitted) and harvested at late-logarithmic phase. LPS was prepared by the method of Galanos et al. (1969) and was quantified by the heptose assay (Osborn, 1963). Following the current concept of LPS structure (Nikaido & Nakae, 1979), we assume that 1 mol of LPS contains 3 mol of heptose. Phospholipids were extracted from *E. coli* B by the method of Folch et al. (1957). Phosphorus was assayed according to Ames & Dubin (1960). Porin was purified from *E. coli* B as trimers according to Tokunaga et al. (1979) and was quantitated by the protein assay (Lowry et al., 1951).

Mixed Bilayer Preparation from LPS and Phospholipids. In order to study the diffusion of phospholipid molecules into LPS domains, a spin-labeled phospholipid, PE* or PG* (30 nmol), was dried up from its benzene solutions in a 12 × 100 mm test tube under a stream of N_2. An aqueous suspension of LPS (50–500 nmol) was added onto the film of phospholipid spin-label. The mixture had a total volume of 0.2 mL and contained in addition 10 mM Hepes–NaOH (pH 7.2) with or without 10 mM $MgCl_2$, and with or without 0.2 M NaCl. It was sonicated with a Braunsonic 1510 sonicator with a microprobe for two 30-s periods at the minimum power setting in an ice bath. ESR measurements were performed immediately after sonication, and then the samples were incubated at 37 °C. At indicated times, portions of the samples were examined by the ESR technique. In experiments where low LPS/labeled phospholipid ratios were employed, controls were run in which LPS was replaced by unlabeled *E. coli* B phospholipids containing the same number of fatty acid residues. In these cases, the unlabeled and labeled phospholipids were mixed together in benzene, dried as a film, and resuspended by sonication in an appropriate buffer.

In another series of experiments, *E. coli* B phospholipids (3 μmol) and spin-labeled phospholipid (30 nmol) were dried up together from their benzene solutions. Aqueous dispersions were prepared as described above in the presence or absence of LPS (500 nmol).

Isopycnic Centrifugation of Lipid Dispersions. Dispersions were made, in 0.2 *M* NaCl–0.02 M $MgCl_2$, from 90 nmol of PE* and either 150 nmol of LPS or 450 nmol of *E. coli* phospholipids, in a total volume of 0.2 mL. The suspensions were layered on a step gradient of sucrose (20, 30, 40, 50, and 60% w/v) in 0.2 M NaCl–0.01 M $MgCl_2$ (total volume, 0.65 mL in a Beckman 305528 tube), and the tubes were centrifuged in a Beckman SW65 rotor with adapters at 170000*g* for 13 h at 2 °C. The content of the tube was collected in four fractions, plus the resuspended pellet, each about 0.16 mL, and ESR spectra were determined on each fraction. Spin-labels in each fraction were quantitated by integrating the first-derivative spectra twice by a computer, as described by Jost & Griffith (1976).

Other Methods. All ESR measurements were made at room temperature (about 23 °C) with a Varian V-4500 spectrometer. Electrodialysis was performed according to Galanos et al. (1969).

Results

Principles of the Approach. In the experiments of the first type, we used a modification of the method of Devaux & McConnell (1972) (Figure 1a). Mixed bilayers were made from pure spin-labeled phospholipids and different amounts of LPS. At the beginning, the phospholipids and LPS were expected to exist largely in separate domains. If so, the ESR spectra would show an extensive broadening, owing to the strong spin exchange and dipole interactions between nitroxide

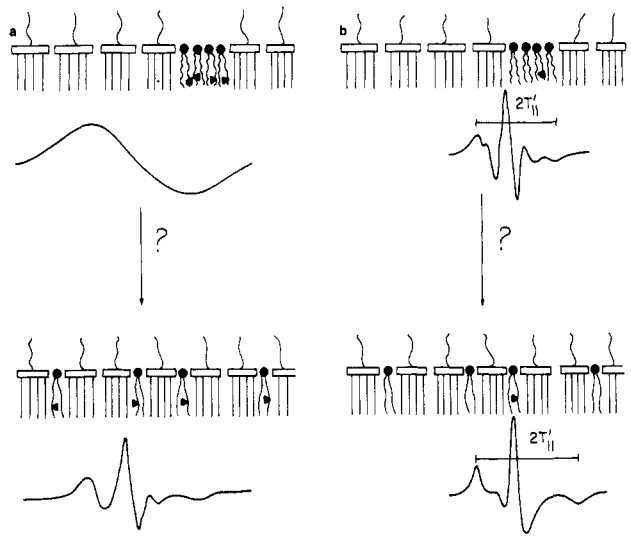

FIGURE 1: Principles of the approach. This figure shows schematically the expected structures of various mixed bilayer preparations and corresponding ESR spectra. For simplicity, only half of the bilayer is shown. The molecule containing several hydrocarbon chains is LPS. The filled-in triangles (▲) represent the 4,4-dimethyloxazolidinyl-*N*-oxy spin-label groups. (a) In this approach, the close proximity between spin-labeled phospholipids in the phospholipid domains initially produces extensive line broadening. If the labeled phospholipids diffuse into LPS domains, the average distance between labels will increase, resulting in the narrowing of the ESR line widths. (b) In the second approach, the labeled phospholipids are diluted with unlabeled phospholipids. There is little exchange broadening, and the label reports mainly the local fluidity. If the phospholipids (including the labeled ones) diffuse into the LPS domains, the mobility of the label should become more restricted, resulting in the increase in the maximum hyperfine splitting ($2T_{\parallel}'$) values. For details see text.

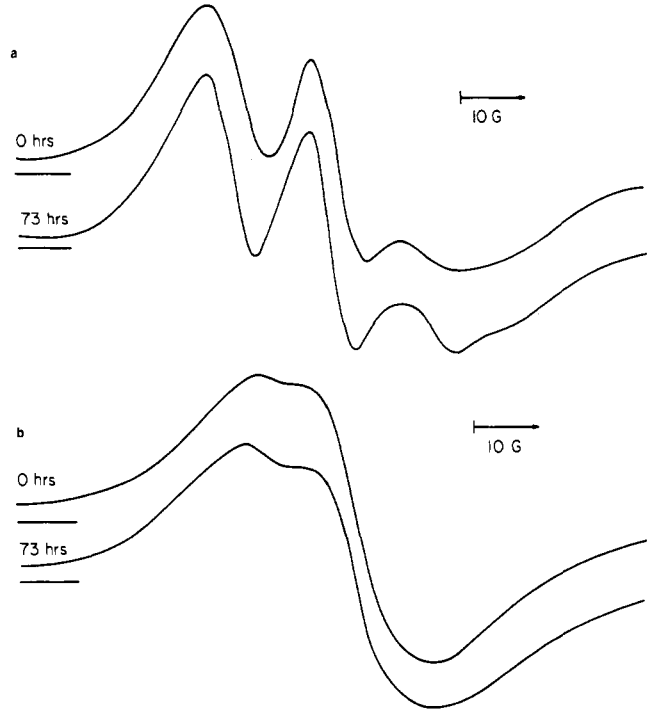

FIGURE 2: ESR spectra of PE*–phospholipids and PE*–LPS dispersions. Aqueous dispersions containing 30 nmol of PE* and either 150 nmol of *E. coli* B phospholipids (300 nmol of fatty acid residues) (a) or 50 nmol of *E. coli* B LPS (300 nmol of fatty acid residues) (b) were made in 0.2 M NaCl–0.01 M $MgCl_2$ as described under Materials and Methods. The spectra shown are those obtained after 0 and 73 h of incubation at 37 °C.

radicals located closed to each other. If the labeled phospholipids gradually diffused into the areas in between LPS molecules as shown in Figure 1a, the increase in distance between the labeled molecules would result in the narrowing of the line width of the ESR spectra. The decrease in line width can, therefore, be used to follow the process of mixing of the domains.

In the experiments of the second type (Figure 1b), the spin-labeled phospholipids were diluted by an excess of unlabeled phospholipids, and after the mixed phospholipids were added to LPS, the degree of mobility of the hydrocarbon chains of the labeled phospholipids was examined by measuring the magnitude of maximum hyperfine splitting in the ESR spectra. Since LPS hydrocarbon chains are less mobile and expected to have an immobilizing effect on hydrocarbon chains located nearby (Nikaido et al., 1977; see also below), the intermixing of phospholipid and LPS domains would cause the progressive immobilization of the probe, i.e., increase in maximum hyperfine splitting.

Mixtures of Spin-Labeled Phospholipids and LPS at "Physiological" Ratios. In these experiments, we mixed PE* (or PG*) with LPS, at ratios not far from the molar ratio of phospholipids to LPS in the outer membrane [about 1:0.3 according to Smit et al. (1975)]. Under these conditions, some line broadening will remain even after the complete mixing of labeled phospholipids with LPS. We therefore mixed PG* or PE* with the total lipids of *E. coli* containing approximately the same numbers of fatty acid residues as LPS; these samples indicated roughly the degree of narrowing of the line width expected after the complete mixing together of labeled and unlabeled components.

As shown in Figure 2, in control mixtures the line broadening decreased almost to its final equilibrium values already at the earliest time point measurements could be made. In contrast, PE*–LPS mixtures produced strongly broadened spectra even after a 3-day incubation at 37 °C. Similar results were obtained with PG*–LPS vesicles (data not shown).

In order to follow the diffusion or homogenization process in a quantitative manner, we used a parameter, α, introduced by Devaux & McConnell (1972). The spectra with extensive line broadening have small values of α whereas those with no line broadening should have infinitely large values of α.

The time course of the change in α is shown in Figure 3 under various conditions. Clearly, the rates of diffusion of both PE* and PG* were in the undetectable or, at most, barely detectable range. It should be noted that although the diffusion of labeled phospholipids in phospholipid bilayers was also rather slow in the original experiment of Devaux & McConnell (1972) they were measuring the diffusion process across macroscopic distances of the order of several millimeters. In contrast, in our system, the size of the initial domains, and thus the distance that has to be travelled by the diffusing molecules, is very small, certainly much smaller than the average size of vesicles. [Vesicles made under similar conditions had an average diameter of 100 nm (Nakae, 1976).] Indeed, the diffusion coefficient given by Devaux & McConnell (1972) suggests that the mixing will be completed in a few milliseconds in control vesicles containing only phospholipids.

These results therefore indicate that the intermixing of spin-labeled phospholipid domains with those of LPS is a process many orders of magnitude slower than the lateral diffusion of phospholipids in phospholipid bilayers. An alternative explanation is that the LPS and phospholipids exist in separate vesicles, and the slow mixing is primarily a result of the vesicle fusion process. However, this appears rather unlikely in view of the following results: (a) The sonication step is at least sufficient to produce homogeneous mixed vesicles containing both PE* (or PG*) and unlabeled phos-

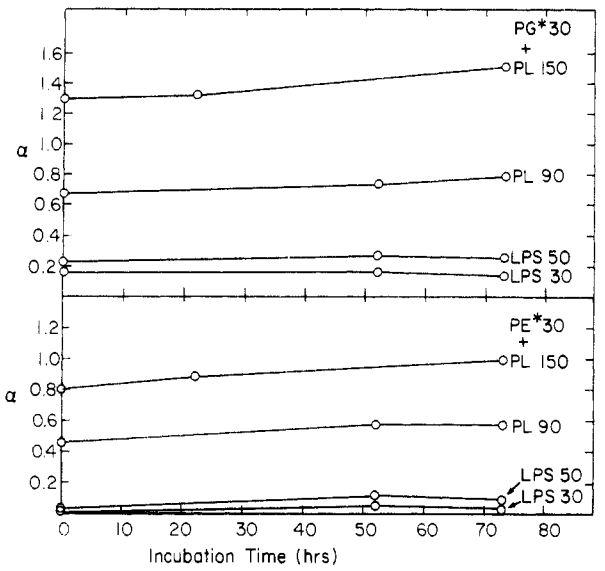

FIGURE 3: Time course of intermixing of phospholipid and LPS domains in vesicles containing phospholipids and LPS in nearly "physiological" ratios. Dispersions were prepared from 30 nmol of PE* (or PG*) and various amounts of phospholipids and LPS in 0.2 M NaCl–0.01 M $MgCl_2$ as described under Materials and Methods, incubated at 37 °C, and at times indicated used for the determination of ESR spectra, from which parameter α (Devaux & McConnell, 1972) was calculated. The amounts of phospholipids and LPS (in nmol) are shown in the figure. Since a molecule of LPS contains 3 times as many fatty acid chains as a molecule of phospholipid, PL 90 and PL 150, respectively, serve as controls for LPS 30 and LPS 50 and show roughly the extent of line narrowing (as expressed by the magnitude of α parameter) attainable if complete mixing of LPS and PE* (or PG*) molecules were achieved. Although the PE* (or PG*) plus phospholipid vesicles in this experiment were made by mixing the labeled and unlabeled lipids in an organic solvent (see Materials and Methods), identical results were obtained by adding an aqueous suspension of phospholipid liposomes to films of PE* (or PG*) and sonicating the mixture, i.e., by preparing the control dispersions in exactly the same manner as we prepared the PE* (PG*) plus LPS dispersions (results not shown).

pholipids (see legend to Figure 3). (b) Isopycnic sucrose density gradient centrifugation of the PE*–LPS mixture immediately after its preparation (see Materials and Methods) showed that more than 90% of the spin label, as detected by ESR measurement, was located in the bottom fraction and the pellet whereas PE*–phospholipids dispersion was recovered entirely in the top fraction (not shown). Although the aggregation of vesicles can never be completely ruled out, the recovery of the PE*–LPS dispersions with a narrow range of buoyant density strongly suggests the presence of a rather homogeneous population of mixed vesicles.

Mixed Bilayers Containing Unlabeled Phospholipids. In the experiments described above, the only phospholipids present in the system were spin-labeled phospholipids. We cannot, therefore, rule out the possibility that the unexpectedly slow diffusion of PE* and PG* was due to unusually strong interactions between these "unnatural" molecules. Furthermore, the extent of exchange broadening is affected by the fluidity of the hydrocarbon domains (Sackmann & Träuble, 1972); therefore, it can be argued that the phospholipid–PE* (or PG*) mixtures in Figure 3 are not the ideal controls for LPS–PE* (or PG*) mixtures. Finally, it is possible that initially many spin-labeled phospholipid molecules became associated, in a "cooperative" manner, with just a few LPS molecules and produced significant broadening due to spin–spin interactions.

We carried out experiments of the second type in order to rule out these possibilities. In these experiments, the "fluidity" or the mobility of phospholipid hydrocarbon chains, rather than

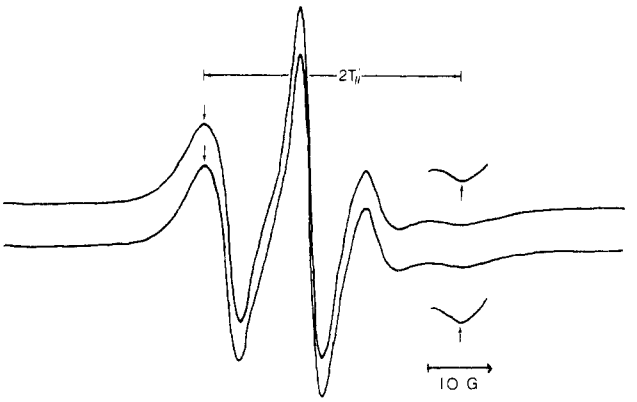

FIGURE 4: ESR spectra of dispersions containing a PE*–phospholipids–LPS mixture (above) and a PE*–phospholipids mixture (below). PE* (30 nmol) was mixed either with *E. coli* phospholipids (3 μmol) and LPS (500 nmol) or with phospholipids (3 μmol) in 10 mM Hepes–NaOH, pH 7.2, and vesicles were incubated at 37 °C. The spectra shown are those after 3 days at 37 °C, but no detectable change was observed in either system during this period of incubation. The spectra were not altered by an inclusion of 0.2 M NaCl, 0.01 M $MgCl_2$, or both in the vesicle preparation medium. The high-field portions of the spectra were also recorded with a 3-fold increased sensitivity in order to measure the maximum hyperfine splitting ($2T_{\parallel}'$) values accurately. Arrows indicate the positions of high- and low-field peaks used for the determination of $2T_{\parallel}'$.

the line broadening, is used as the index of the intermixing of the domains (Figure 1b).

When PE* or PG* was mixed with *E. coli* total lipid in a molar ratio of 1:100, both spin-labeled phospholipids showed very similar values of maximum hyperfine splitting, approximately 40 G (Figure 4), a result indicating a rapid anisotropic motion of the nitroxide group. This value was not altered by the addition of 10 mM Mg^{2+} or 0.2 M NaCl, or by further incubation of the sample at 37 °C.

As described under the section Principles of the Approach, diffusion of much of the phospholipids into LPS domains should produce significant immobilization of the hydrocarbon chains of phospholipids (Figure 1b). This idea is indeed supported by the observation that both PE* and PG* showed a greatly increased maximum hyperfine splitting value of 51 G when mixed bialyers containing PE* or PG* and a 17-fold molar excess of LPS were incubated at 37 °C for several days. (Data are not shown, but the 3-day sample in Figure 6 shows splitting values not far from this.) When phospholipids containing 1% spin-labeled species and LPS were reconstituted into mixed bilayers at the molar ratio of 6:1 (or the fatty acid residue molar ratio of 2:1), the "mobility" of the phospholipid hydrocarbon chains, as measured by the hyperfine splitting of the ESR spectra, remained exactly the same as in the control bilayer containing phospholipid only, and there was no progressive immobilization even after a 3-day incubation at 37 °C (Figure 4).

These data indicate that there was no detectable mixing between LPS and phospholipid domains. We emphasize that these data do not suffer from the alternative possibilities discussed earlier in this section. Here we are not dealing with the interaction between molecules of spin-labeled phospholipids. Furthermore, a tight association between many phospholipid molecules and a single LPS molecule should have produced large increases in hyperfine splitting.

These results should also be compared to our previous results (Nikaido et al., 1977). Thus, in the previous experiments, phospholipid–LPS mixed bilayers showed significantly reduced fluidity in comparison with the bilayers containing phospholipids alone whereas an identical value of hyperfine splitting

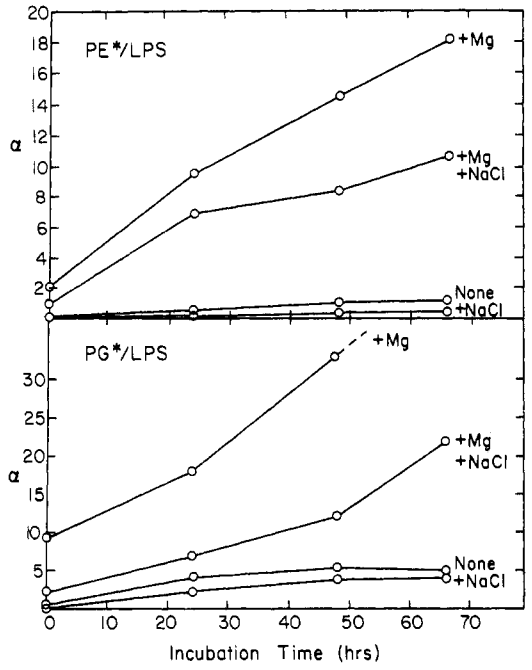

FIGURE 5: Kinetics of diffusion of PE* (above) and PG* (below) into LPS domains in dispersions containing a large excess of LPS. Lipid dispersions were prepared from 30 nmol of PE* (or PG*) and 500 nmol of LPS in 10 mM Hepes–NaOH buffer, pH 7.2, with or without 0.2 M NaCl and 0.01 M $MgCl_2$, as described under Materials and Methods. After incubation for indicated periods, ESR spectra were taken, and parameter α was calculated as in Figure 3.

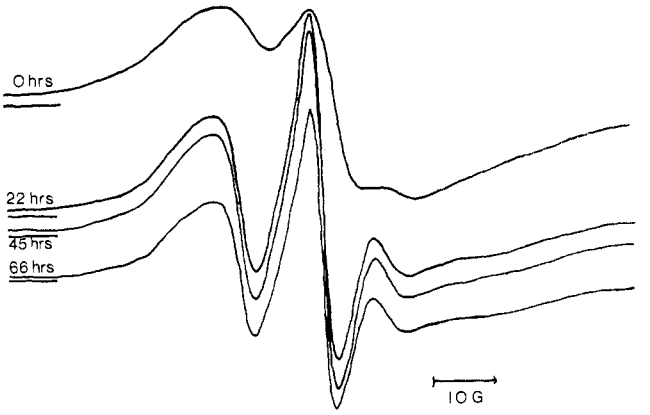

FIGURE 6: ESR spectra of PE*–LPS dispersions. This shows the actual spectra of PE*–LPS (30–500 nmol) dispersions made in 10 mM Hepes–NaOH, pH 7.2, containing 10 mM $MgCl_2$, incubated at 37 °C. For details, see Materials and Methods.

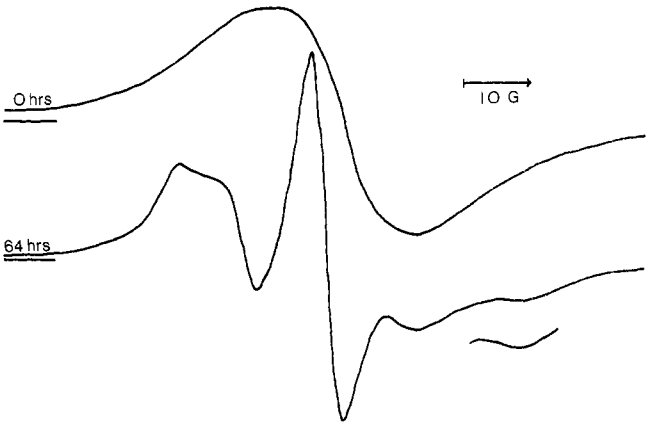

FIGURE 7: ESR spectra of PE*–LPS dispersions. This shows the actual spectra of PE*–LPS (30–500 nmol) dispersions made in 10 mM Hepes–NaOH, pH 7.2, not containing $MgCl_2$ or NaCl. Only the spectra obtained at 0 and 64 h are shown for simplicity.

was observed in these two types of bilayers in the present experiments. There is no real discrepancy, however, between these results when we consider that the spin-labeled probe used in the earlier experiments was a free fatty acid, which most probably sampled the fluidity of both phospholipid and LPS domains present in the mixed bilayer. [In fact, we could confirm our previous results by using 5-nitroxide stearate as the probe (data not shown).] In contrast, the phospholipid spin label used in this investigation was presumably confined to the phospholipid domains, which apparently persisted as independent domains even after 3 days.

Mixture of Spin-Labeled Phospholipids and a Large Excess of LPS. In an effort to accelerate the process of diffusion of phospholipids, we repeated the experiments of the first type (Figure 1a) by using much larger amounts of LPS (molar ratio of labeled phospholipid to LPS, 1:17). Under these conditions, complete intermixing will produce an extensive dilution of labeled molecules and practically abolish the line broadening of the ESR spectra. Thus, controls with unlabeled phospholipids were not necessary.

As seen in Figure 5, both PE* and PG* showed measurable rates of diffusion under these extreme conditions. In an effort to probe the mechanism of this slow diffusion process, various conditions were tested. With PE*, Mg^{2+} stimulated the diffusion whereas 0.2 M NaCl inhibited the process. PG* diffused, in general, more rapidly than PE*, and NaCl retarded the process. The effect of Mg^{2+} was not reproducible in the PG*–LPS system, stimulatory with one preparation of LPS (shown in Figure 5), and somewhat inhibitory with another (not shown). Since both PG* and LPS are negatively charged, it seems possible that electrostatic forces play a major part in the mixing process and that the effect of Mg^{2+} is influenced by how much Mg^{2+}, cationic groups, and anionic groups are present in a given LPS preparation.

The ESR spectra of the PE*–LPS mixture in 10 mM $MgCl_2$ are shown in Figure 6. The progressive decrease in line broadening is evident. The spectra of the mixture in other solutions are not shown, except the one obtained after a 3-day incubation in the absence of Mg^{2+} and NaCl (Figure 7). Comparison of the spectrum of the 66-h sample in Figure 6 with that of a similar sample in Figure 7 reveals that the latter contains at least two components, one of which is more strongly immobilized than the PE* molecules in the sample in Figure 6. When a sample similar to the 66-h sample of Figure 6 was subjected to electrodialysis in order to remove Mg^{2+}, there was an appearance of an immobilized component similar to that of Figure 7 (results not shown). Rather surprisingly, therefore, the absence of added Mg^{2+} appears to result in the immobilization of some of the PE* molecules, which might be surrounded by LPS. Similar results were also obtained with PG*–LPS dispersions.

Effects of Porin on the Mixing of Domains. Porin is usually the most abundant protein in the outer membrane of *E. coli* (Nikaido & Nakae, 1979) and is reported to have strong interactions with LPS (Yu & Mizushima, 1977). It was desirable, therefore, to examine the effect of porin on the mixing of phospholipids and LPS domains.

When 100 μg of purified porin from *E. coli* B was added to the "physiological ratio mixture" of 30 nmol of PE* and 30 nmol of LPS, there was little intermixing of domains even after 3 days at 37 °C (not shown). However, when the same amount of porin was added to a mixture of spin-labeled phospholipids and a large excess of LPS (molar ratio 1:17), some stimulatory effects were observed (Figure 8) although

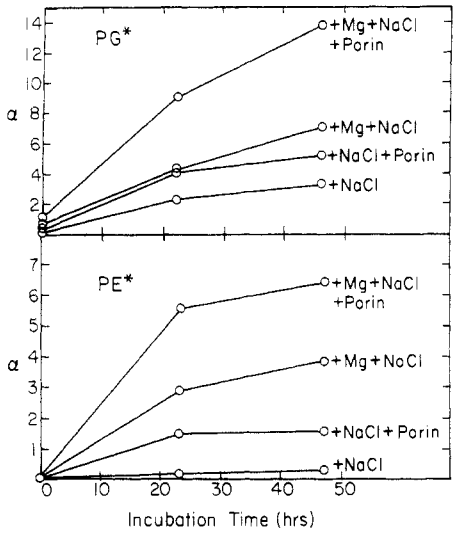

FIGURE 8: Effect of porin on the rate of diffusion of phospholipids into LPS domains. Dispersions were made from 30 nmol of PG* (or PE*) and 500 nmol of LPS. To some tubes was also added porin (100 μg), NaCl (0.2 M), or $MgCl_2$ (0.01 M). After incubation at 37 °C for the indicated time, the ESR spectrum was obtained, and parameter α was calculated. For details, see Materials and Methods.

complete equilibration still appeared to require many days. This effect is not due to the detergent sodium dodecyl sulfate ($NaDodSO_4$), which exists in trace amounts in these porin preparations. According to a previous study from our laboratory (Nakae, 1976), they contain less than 0.1 μg of $NaDodSO_4$ per 100 μg of porin, yet up to 1 μg of $NaDodSO_4$ did not produce any significant changes in the rate of diffusion of PE* and PG* in systems containing 17-fold molar excesses of LPS (data not shown).

Discussion

The major finding of this study was that the lateral diffusion of phospholipid molecules into LPS domains, or the intermixing of phospholipid and LPS domains, was an extremely slow process in comparison with the rates of lateral diffusion of phospholipids in phospholipid bilayers. Since this observation was rather unexpected, we tried various conditions in the hope of finding one that accelerated the process significantly. Although we did find that Mg^{2+} and porin increased the rate of phospholipid diffusion in the presence of an overwhelming excess of LPS, the process still remained relatively slow even under the optimal conditions.

Because these results are at first sight somewhat contradictory to the impression generated by the studies of Rothfield's group (Weiser & Rothfield, 1968; Romeo et al., 1970; Rothfield & Romeo, 1971; Fried & Rothfield, 1978), that phospholipids, especially PE, very strongly interact with LPS, we shall carefully compare our results with those of Rothfield and his associates.

First, many of the previous studies showing facile insertion of LPS into phospholipid domains have been done with monolayers (Romeo et al., 1970; Fried & Rothfield, 1978). Possibly, monolayers are very different from bilayers or biomembranes because the former lack, at least under conditions of low film pressure, the strong cohesive interactions between the hydrocarbon chains of the phospholipids, as well as tight interactions between the head groups of neighboring molecules. Perhaps these conditions facilitate the insertion of LPS molecules and allow them to interact with PE without the constraints imposed in bilayer studies. Second, with bilayers, Rothfield's group has found it necessary to keep the mixed vesicles at 60 °C for 30 min in order to produce homogeneous vesicles (Weiser & Rothfield, 1968). This cannot be due simply to the requirement for the fluid state of the LPS or phospholipid hydrocarbon chains since they appear to be completely fluid above 25 °C (Emmerling et al., 1977) and 10 °C (Melchior & Steim, 1976), respectively. We feel that this requirement for heating is more likely to be related to the need to speed up the inherently slow process of diffusion of phospholipids into LPS domains. We have not measured diffusion rates at 60 °C, but if, as expected, the diffusion becomes much faster at 60 °C perhaps there is no real discrepancy between our results and those of Weiser & Rothfield (1968). Finally, in some of the experiments of Rothfield's group (Fried & Rothfield, 1978), what is measured is mainly the strength of interaction between LPS and phospholipids whereas in our study we are measuring the balance between the cohesive interaction between LPS and phospholipid molecules and the sum of LPS-to-LPS and phospholipid-to-phospholipid interactions. Thus, much of the disagreement between our and Rothfield's results could actually be illusory.

Our results also seem to form a contrast with the recent work of Schindler et al. (1980), who found, from the kinetics of recovery after photobleaching of multiple bilayer preparations, that a fluorescent derivative of PE diffused rather rapidly in PE–LPS mixed bilayers, with a diffusion coefficient only 1 order of magnitude slower than that in phospholipid bilayers. However, again a real discrepancy might not exist, because in view of our results what they studied might have been the lateral diffusion of the phospholipid domains rather than that of individual phospholipid molecules. We note that, according to the theoretical analysis of Saffman & Delbrück (1975), even domains containing 10^4 molecules of phospholipids would diffuse with a diffusion coefficient only 1 order of magnitude smaller than that for individual phospholipids. Furthermore, in the study of Schindler et al. (1980), all PE and LPS molecules have been modified by the introduction of very large fluorescent reporter groups, a procedure which could have drastically altered the strength of interaction between these molecules. Such criticisms do not apply to our experiments of at least the second type.

In dispersions containing LPS and phospholipids at "physiological" ratios, the diffusion of phospholipids into LPS domains was hardly detectable even after a 3-day incubation at 37 °C (Figures 3 and 4). Thus, it is possible that the coexistence of phospholipid domains and LPS represents the state of thermodynamic equilibrium. In fact, Wu & McConnell (1975) found that 1,2-dielaidoyl-*sn*-glycero-3-phosphorylcholine and 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylethanolamine coexisted as separate, immiscible fluid domains in a mixed bilayer under certain conditions. This idea is attractive because, as predicted by Wu & McConnell (1975), such a phase separation tends to produce, spontaneously, asymmetric bilayers with each species of the lipids predominantly on one side of the membrane, precisely the situation in the outer membrane (Kamio & Nikaido, 1976) (see also below). Mixing of phospholipids and LPS did occur when small amounts of phospholipids were mixed with a large excess of LPS (Figures 5 and 8). This is probably analogous to the situation in the binary mixture of partially miscible liquids (e.g., phenol and water), where a single phase solution results if an overwhelming excess of one component is present.

An alternative interpretation of the results is that the coexistence of the segregated phases is not a thermodynamically most stable state, but the approach to the fully mixed, equilibrium state is prevented kinetically, i.e., by the slow rates of the mixing process. Our data at present do not allow us

to choose between these two possibilities.

We hoped that the study of parameters affecting the diffusion rates might shed some light on the mechanism of the mixing process. However, the results do not seem to lead to any clear-cut interpretation; we simply need to know much more about the molecular details of interaction between Mg^{2+}, NaCl, or porin on one hand and phospholipids and LPS on the other. We have consistently observed that PE diffused much more slowly into LPS domains than PG did. Although this is unexpected in view of published results (Rothfield & Romeo, 1971) suggesting the special affinity between PE and LPS, we cannot conclude anything about the relative strengths of PE–LPS and PG–LPS interactions, because what affected our results is most probably the balance or difference between various types of interactions, as described already. In the case of PG, the phospholipid-to-phospholipid interaction is probably weak because of the repulsive forces between the negatively charged head groups, and possibly the weakness of this interaction played a major role in the diffusion of PG molecules into the LPS domains.

Whatever the underlying mechanism, this stable coexistence of LPS domains and phospholipid domains in mixed bilayers could be advantageous for the bacterial cell. First, this could help the assembly of the outer membrane. Thus, the LPS molecules are synthesized in the cytoplasmic membrane. At this stage, the few LPS molecules being synthesized are held onto the membrane, presumably by the strong PE-to-LPS interaction (Rothfield & Romeo, 1971). As more and more LPS molecules are synthesized, they could become clustered and form an independent domain. Such pure LPS domains could then be exported, perhaps via lateral diffusion or the membrane fusion process [see Figure 3c,d of Osborn (1979)], to become ultimately the outer leaflet of the outer membrane. This mechanism explains how the pure LPS domains of the outer membrane (Nikaido et al., 1977) could be assembled in spite of the presence of the supposedly strong PE-to-LPS interactions. Second, the stability of the LPS domains and phospholipid domains would be an advantage in maintaining the highly asymmetric architecture of the outer membrane, where most, if not all, of LPS and phospholipids are segregated into the outer and inner leaflets, respectively (Mühlradt & Golecki, 1975; Kamio & Nikaido, 1976; Funahara & Nikaido, 1980). Thus, the occasional appearance of phospholipid molecules in the outer leaflet, by "flip-flop", fusion, or any other mechanism, still would not produce an instantaneous randomization of the distribution of the lipid components of the cell surface.

Finally, we note that the separation of lipid domains, if it indeed corresponds to the thermodynamically most stable state, may have important implications on the origin of asymmetry of various biological membranes. Hitherto it has been customary to assume that the asymmetric distribution of lipid components was caused by the inherent asymmetry in the structure of transmembrane proteins because lipids were thought to form a homogeneous mixed phase if left alone by themselves. But if lipids could spontaneously segregate into separate domains and form asymmetric bilayers as predicted by Wu & McConnell (1975), the possibility that such a process plays a major, and even the primary, role in the generation of membrane asymmetry should be seriously considered.

Acknowledgments

We are very grateful to Dr. W. Hubbell of the Department of Chemistry for allowing us to use his Varian spectrometer and to Dr. A. N. Glazer of this department for his comments on the manuscript.

References

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769–775.

Bertani, G. (1951) *J. Bacteriol. 62*, 293–300.

Devaux, P., & McConnell, H. M. (1972) *J. Am. Chem. Soc. 94*, 4475–4481.

Emmerling, G., Henning, U., & Gulik-Krzywicki, T. (1977) *Eur. J. Biochem. 78*, 503–509.

Folch, J., Lees, M., & Stanley, G. H. S. (1957) *J. Biol. Chem. 226*, 497–509.

Fried, V. A., & Rothfield, L. (1978) *Biochim. Biophys. Acta 514*, 69–82.

Funahara, Y., & Nikaido, H. (1980) *J. Bacteriol. 141*, 1463–1465.

Galanos, C., Lüderitz, O., & Westphal, O. (1969) *Eur. J. Biochem. 9*, 245–249.

Hubbell, W. L., & McConnell, H. M. (1971) *J. Am. Chem. Soc. 93*, 314–326.

Jost, P., & Griffith, O. H. (1976) in *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) pp 251–272, Academic Press, New York.

Kamio, Y., & Nikaido, H. (1976) *Biochemistry 15*, 2561–2570.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Melchior, D. L., & Steim, J. M. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 205–238.

Mühlradt, P. F., & Golecki, J. R. (1975) *Eur. J. Biochem. 51*, 343–352.

Nakae, T. (1976) *J. Biol. Chem. 251*, 2176–2178.

Nikaido, H., & Nakae, T. (1979) *Adv. Microb. Physiol. 20*, 163–250.

Nikaido, H., Takeuchi, Y., Ohnishi, S.-I., & Nakae, T. (1977) *Biochim. Biophys. Acta 465*, 152–164.

Osborn, M. J. (1963) *Proc. Natl. Acad. Sci. U.S.A. 50*, 499–506.

Osborn, M. J. (1979) in *Bacterial Outer Membranes* (Inouye, M., Ed.) pp 15–34, Wiley-Interscience, New York.

Osborn, M. J., Gander, J. E., Parisi, E., & Carson, J. (1972) *J. Biol. Chem. 247*, 3962–3972.

Romeo, D., Girard, A., & Rothfield, L. (1970) *J. Mol. Biol. 53*, 475–490.

Rothfield, L., & Romeo, D. (1971) *Bacteriol. Rev. 35*, 14–38.

Sackmann, E., & Träuble, H. (1972) *J. Am. Chem. Soc. 94*, 4482–4491.

Saffman, P. G., & Delbrück, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3111–3113.

Schindler, M., Osborn, M. J., & Koppel, D. E. (1980) *Nature (London) 283*, 346–350.

Smit, J., Kamio, Y., & Nikaido, H. (1975) *J. Bacteriol. 124*, 942–958.

Takeuchi, Y., Ohnishi, S.-I., Ishinaga, M., & Kito, M. (1978) *Biochim. Biophys. Acta 506*, 54–63.

Tokunata, M., Tokunata, H., Okajima, Y., & Nakae, T. (1979) *Eur. J. Biochem. 95*, 441–448.

Weiser, M. M., & Rothfield, L. (1968) *J. Biol. Chem. 243*, 1320–1328.

Wu, S. H., & McConnell, H. M. (1975) *Biochemistry 14*, 847–854.

Yu, F., & Mizushima, S. (1977) *Biochem. Biophys. Res. Commun. 74*, 1397–1402.