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# OUTER MEMBRANE OF *SALMONELLA TYPHIMURIUM*

# ELECTRON SPIN RESONANCE STUDIES *

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## Summary

The supramolecular structure of the outer membrane of *Salmonella typhimurium* that produces an Rc-type lipopolysaccharide was studied by adding spin-labeled fatty acid probes to membranes as well as model bilayers. Lipopolysaccharide of this organism apparently formed a bilayer structure in 0.2 M NaCl/0.01 M $MgCl_2$, and the electron spin resonance spectra suggested that the motion of the segments of hydrocarbon chains near the carboxyl end was quite restricted even at high temperature; this is presumably due to the anchoring of more than a dozen fatty acid residues to a single backbone structure. In the presence of $Mg^{2+}$, we could produce lipopolysaccharide-phospholipid mixed bilayers containing up to 50% (by weight) lipopolysaccharide. Their spectra showed no sign of major heterogeneity, and the maximum hyperfine splitting values were considerably larger than in phospholipid-only liposomes; these results suggest that the two components are finely interspersed and that the mobility of phospholipid hydrocarbons is severely restricted by the hydrocarbon chains of lipopolysaccharide. In spite of the presence of lipopolysaccharide in an amount equal to or exceeding that of phospholipids, the outer membrane produced spectra remarkably similar to those of the inner membrane, which does not contain lipopolysaccharide, and there was little sign of immobilization by lipopolysaccharides. Signals corresponding to the pure lipopolysaccharide phase were not detected, either. These results suggest that the phospholipids and lipopolysaccharides are segregated into separate domains in the outer

* This is paper XIII of the series on the outer membrane of bacteria.

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membrane, and the fatty acid probes enter almost exclusively into the phospholipid domains. This conclusion was fully corroborated by determining, through the exchange broadening of line width, the total area of the domains that accommodated the spin label probes.

## Introduction

Cells of Gram-negative bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, are surrounded by the cytoplasmic (or "inner") membrane, as well as by an outer membrane, which contains proteins, phospholipids, and lipopolysaccharides and forms a part of the cell wall [1]. Lipopolysaccharide is an amphipathic substance and contains about 18 saturated fatty acid residues attached covalently at one end of the molecule (see Fig. 1). Lipopolysaccharides and phospholipids easily form mixed bilayers [2] and mixed monolayers [3]. It has therefore been generally assumed that the continuum of the outer membrane is made up of the mixed bilayer, i.e. a phospholipid bilayer with interdigitating lipopolysaccharide molecules [1,4—7]. More recently, however, it has become increasingly clear that the outer membrane is extremely asymmetric in structure. Thus lipopolysaccharide was shown to be located only in the outer half of the membrane [8], and non-penetrating agents such as *Bacillus cereus* phospholipase C and CNBr-activated dextran failed to reveal exposed phospholipid head groups on the outer surface of bacteria [9], even in "Rc"

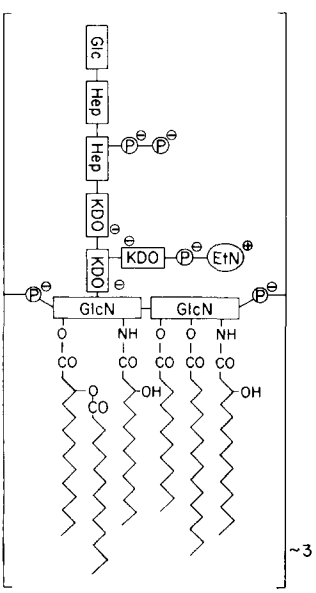


Fig. 1. Structure of lipopolysaccharide from HN202. Each rectangle represents a sugar residue (Glc, glucose; Hep, L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-D-*manno*-octulosonic acid; GlcN, D-glucosamine). Experimental findings that led to this structure are cited in ref. 1 Note that the precise location of individual, ester-linked fatty acid chains is not known, and the arrangement here is arbitrary. Other abbreviations: P, phosphate; EtN, ethanolamine.

mutants producing quite defective lipopolysaccharide with only six sugar residues in the "polysaccharide" chain (see Fig. 1). The latter results can be most easily explained by assuming the presence of phospholipid molecules only in the inner half of the outer membrane, and this interpretation suggests that there may be a complete segregation of lipopolysaccharides and phospholipids into the outer and inner halves, respectively, of the outer membrane, which therefore may not contain any mixed bilayer regions at all. In this paper, the molecular organization of the outer membrane has been studied by using spin-labeled probes. The results are consistent with the hypothesis described above and suggest complete separation of domains occupied by lipopolysaccharides from those occupied by phospholipids.

## Materials and Methods

*Bacterial strain. S. typhimurium* HN202 (LT2 *galE503)* was used throughout. This mutant, which corresponds to strain M1 of ref. 10, produces Rc-type lipopolysaccharide with a short "polysaccharide" chain containing only six sugar residues (Fig. 1) instead of more than 40 sugar residues found in the wild type. This mutant was used because interactions between the polysaccharide chains of lipopolysaccharide were unlikely, and yet the interactions of the lipopolysaccharide with other components of the outer membrane were apparently "normal", as suggested by the fact that the molar ratio of lipopolysaccharide : phospholipid : protein in the outer membrane was very close to that found in the wild type [11].

*Preparation of outer and inner membranes.* Bacteria were grown and membranes were prepared as described previously [11], except that the cells were not frozen, and membranes were kept on ice and were used within 24 h after preparation. Thin-layer chromatography of lipids extracted from the membranes showed that less than 5% of phospholipids had been converted to lysophosphatides during the preparation and storage of the membranes.

*Preparation of lipids and lipopolysaccharides.* Bacteria used for this purpose were grown on meat extract agar overnight at 37° C, scraped off the surface of the agar, and washed with cold distilled water twice. Total lipids were extracted essentially according to the Folch procedure [12], washed once with the "theoretical upper phase mixture" [12], dried over $Na_2SO_4$, and stored at −20° C as a solution in chloroform/methanol (2 : 1, v/v). Such crude lipid preparations from *S. typhimurium* are known to consist mostly of phospholipids (90%) [13].

Lipopolysaccharide was isolated according to Galanos et al. [14], and was kept as a dispersion in distilled water at 4° C after a brief sonication. Its dry weight was determined after drying in vacuo over $P_2O_5$, and the content of fatty acid chains was calculated by assuming that Rc-type lipopolysaccharide, 10 230 daltons [3], contains 18 acyl residues [1].

*Preparation of liposomes and lipopolysaccharide-lipid liposomes.* Liposomes were prepared by drying the *S. typhimurium* lipids as a thin film at the bottom of a test tube and dispersing them in aqueous media. Since the medium of our choice contained $Mg^{2+}$ which tended to cause rapid aggregation of lipids, the lipid film was usually first dispersed in distilled water on a Vortex mixer with

the help of glass beads. NaCl and $MgCl_2$ were then added to make the final concentrations 0.2 and 0.01 M, respectively, and the dispersion (0.4 ml containing 10 $\mu$mol phospholipids) was sonicated for 2—4 periods (each 30 s) with the microtip (diameter: 3 mm) of a probe-type sonicator. In some experiments the suspension was centrifuged, and the thick slurry obtained by suspending the pellet in a minimal amount of supernatant was used for ESR spectroscopy. Mixed lipopolysaccharide-lipid bilayers were produced by a similar method, except that a suspension of lipopolysaccharide in 0.2 M NaCl/0.01 M $MgCl_2$ was used to disperse phospholipid films [15]. In one experiment, the lipids were dispersed in lipopolysaccharide suspended in water, 0.2 M NaCl, or 0.2 M NaCl/0.01 M $MgCl_2$ and each dispersion was layered on top of a 10—30% sucrose gradient (4.0 ml), which in turn was overlayed on top of a 0.5 ml cushion of 60% sucrose [16]. (The sucrose solutions were prepared in water, 0.2 M NaCl, or 0.2 M NaCl/0.01 M $MgCl_2$, respectively). The tubes were then centrifuged at 135 000 $\times g$ in an RPS-50 rotor of a Hitachi 55P centrifuge at 4° C for 18 h.

*Electron spin resonance (ESR) spectroscopy.* The spin-labeled probes used were 4′,4′-dimethyloxazolidine-*N*-oxyl derivatives of 5-keto- and 12-keto-stearic acid, synthesized in our laboratory in Kyoto, here called 5-nitroxide stearate and 12-nitroxide stearate, respectively. The probes were incorporated into membranes and model membranes as follows. (i) With lipids and lipopolysaccharide-lipid mixed bilayers, the methanolic solution of the probe was added to the chloroform/methanol solution of lipids, and the mixture was dried before dispersion in aqueous media. (ii) With membranes and lipopolysaccharide, the probes were dried as a thin film at the bottom of a test tube or a conical centrifuge tube. Then the aqueous suspension of membranes or lipopolysaccharide was added to the tube, and the suspension was mixed on a Vortex mixer for 10—20 s. These procedures usually resulted in a nearly complete incorporation of 5-nitroxide stearate into membranes and liposomes as judged by the absence of the ESR signals belonging to "free" 5-nitroxide stearate. With 12-nitroxide stearate, however, small "free" signals were often observed; the height of the low field and high field lines of these signals, however, never exceeded 10% of the height of the mid field line of the signal generated by the label bound to the membrane. All samples for ESR study were dispersed in 0.2 M NaCl/0.01 M $MgCl_2$ (model bilayers), or in 0.2 M NaCl/0.01 M $MgCl_2$/0.01 M *N*-2-hydroxyethyl-piperazine-*N*′-2′-ethanesulfonic acid buffer, pH 7.4 (membranes).

ESR spectra were obtained with a JEOL ME2X spectrometer, equipped with a variable temperature attachment. In most experiments the mobility of the nitroxide radical was assessed by the magnitude of maximum hyperfine splitting values ($2T_{max}$), which become smaller as the mobility of the probe increases in the membrane [17].

For line broadening experiments, the width of the midfield line was measured as described by Sackmann et al. [18]. In these experiments, the sweep width of 20 G was used along with the modulation width of 1 G.

2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) was used in partition experiments as described in the legend to Fig. 6.

*Other methods.* Phospholipids in the membranes were determined as described previously [11].

## Results

*Preparation of model membranes*

Crude lipids of *S. typhimurium* readily formed liposomes when dispersed in aqueous media [15]. X-ray diffraction studies have shown that lipopolysaccharide molecules are arranged as bilayers in water [19], and ESR spectra of 5-nitroxide stearate-labeled lipopolysaccharide dried down on glass plates also suggested that a large portion of lipopolysaccharide molecules was arranged with the fatty acid chains oriented approximately perpendicular to glass surface (Ohnishi, S., unpublished observations). Furthermore, recent electron microscopic studies suggested that lipopolysaccharide existed, especially in the presence of $Mg^{2+}$, as large sheets, which presumably corresponded to bilayers, yet rarely became closed vesicles [20]. In view of these observations we had little hesitation in treating liposomes and lipopolysaccharide dispersions as model bilayers.

Lipopolysaccharide molecules can become inserted into phospholipid bilayers to form mixed bilayers [2]. Weiser and Rothfield [16] found that the maximum amount of Rc-type lipopolysaccharide that could be inserted into phosphatidylethanolamine bilayer in 0.1 M Tris · HCl buffer, pH 8.5, was 30% of the weight of phosphatidylethanolamine. Since the outer membrane of Rc strains contained lipopolysaccharide and phospholipids in approximately 1—1.5 : 1 ratio (w/w) [11,21], it was necessary to incorporate more lipopolysaccharide into the bilayer if we wanted to approximate the proportion in the native outer membrane. We hypothesized that the incorporation was limited under Weiser and Rothfield's conditions primarily because of electrostatic repulsions between lipopolysaccharide molecules that carry multiple negative charges (see Fig. 1). We therefore tried to minimize this effect by adding $Mg^{2+}$ to the medium. Fig. 2 shows that in 0.01 M $MgCl_2$/0.2 M NaCl, lipopolysaccharide was fully incorporated into bilayer membranes of phospholipid liposomes (eluted at the void volume) until the lipopolysaccharide/phospholipid ratio (w/w) reached 1, and above this ratio excess lipopolysaccharide was seen to be eluted in partially included fractions. That the incorporation of this large amount of lipopolysaccharide requires $Mg^{2+}$ is seen in Fig. 3, which shows the results of the sucrose density gradient equilibrium centrifugation of lipopolysaccharide-lipid mixtures (lipopolysaccharide/lipid ratio = 0.7, w/w). Thus large amounts of lipopolysaccharide could not enter the lipopolysaccharide-lipid complex and were sedimented at the bottom in water as well as in 0.2 M NaCl, but not in 0.2 M NaCl/0.01 M $MgCl_2$. We also want to emphasize the fact that the mixed bilayer made in the presence of $Mg^{2+}$ formed a sharp band, an observation suggesting that a homogeneous population of vesicles each containing lipopolysaccharide and phospholipid in the same ratio had been produced.

*ESR spectra of model membranes*

With 5-nitroxide stearate as a probe, spectra suggesting a partial immobilization of the probe were obtained both with lipopolysaccharide and lipid dispersions. As the temperature was increased, the maximum hyperfine splitting decreased without drastic changes in the general shape of the spectra, a result suggesting that the motion of the probe is anisotropically restricted in an envi-

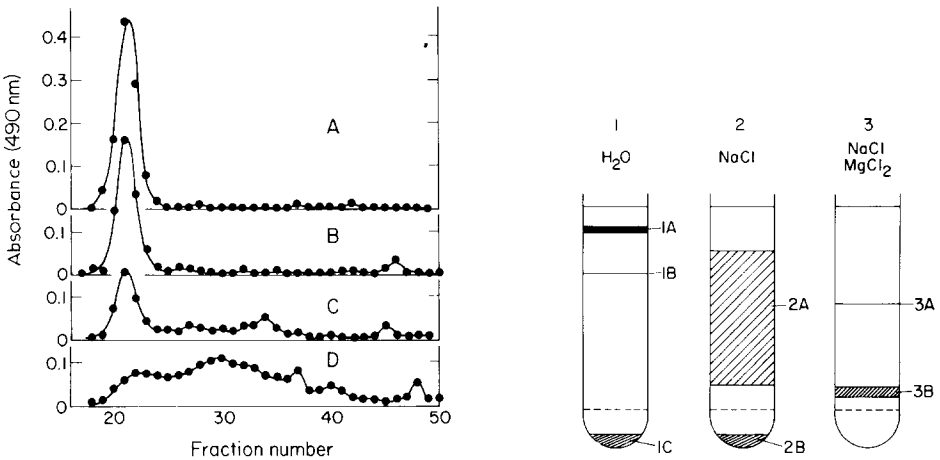


Fig. 2. Gel filtration of lipopolysaccharide complexed with various amounts of lipids. Dispersions of lipids in distilled water were mixed with 1 mg of lipopolysaccharide, dried at the bottom of a test tube under $N_2$ at 45°C, and the film was suspended in 0.2 ml of 0.2 M NaCl/0.01 M $MgCl_2$ with a Vortex mixer. The suspensions were fractionated on a 1.27 × 50 cm column of Sepharose 4B, which had been equilibrated with 0.2 M NaCl/0.01 M $MgCl_2$, and was eluted with the same salt solution. Lipopolysaccharide in each fraction was determined by the phenol-sulfuric acid reaction [22], and the absorbance produced was plotted directly. Samples A, B, C, and D contained, per 1 mg of lipopolysaccharide, 25, 1, 0.35, and 0 mg of lipids, respectively.

Fig. 3. Equilibrium density gradient centrifugation of lipopolysaccharide-lipid complexes. Lipids (4.25 mg) and 5-nitroxide stearate (18 μg) were dried together at bottoms of test tubes. The films were suspended, by vortexing and three cycles of sonication (1 min each), in 0.5 ml dispersions of lipopolysaccharide (2.90 mg) in distilled water, in 0.2 M NaCl, and in 0.2 M NaCl/0.01 M $MgCl_2$. The suspensions were then fractionated by centrifugation through sucrose gradients, as described in Materials and Methods. Broken lines indicate the boundary between the gradient and the cushion of 60% sucrose. ESR spectra suggested that bands 1A and 3A were composed of lipids alone, bands 1C and 2B predominantly of lipopolysaccharide, and bands 2A and 3B of lipopolysaccharide-lipid complexes. Insufficient amount of material was present in band 1B, which could not be studied.

ronment of partially ordered hydrocarbon chains, such as phospholipid or lipopolysaccharide bilayer. We emphasize that the spectra of lipopolysaccharide were very similar to the spectra of lipid dispersions, if compared at temperatures producing similar values of $2T_{max}$. For example, the lipopolysaccharide spectrum at 35.2°C ($2T_{max}$ = 60 G) is almost superimposable on the spectrum of lipid dispersion at 4.6°C ($2T_{max}$ = 60 G), and the lipopolysaccharide spectrum at 47°C ($2T_{max}$ = 57 G) on that of the lipids at 15.0°C ($2T_{max}$ = 56 G) (Fig. 4); these results further support the notion that lipopolysaccharide forms a bilayer-like structure in water.

At any given temperature, however, the maximum hyperfine splitting values were much larger with lipopolysaccharide than with lipids (Fig. 5), suggesting that the mobility of fatty acid chains are more strongly restricted in lipopolysaccharide. Furthermore, the portion of the hydrocarbon chain probed by 5-nitroxide stearate, presumably the portion near the carboxyl end of the chain, does not completely "melt" even at 75°C. This is understandable in view of the fact that in lipopolysaccharide many (18 according to the current concept of lipopolysaccharide structure) fatty acid chains are linked together on a cova-

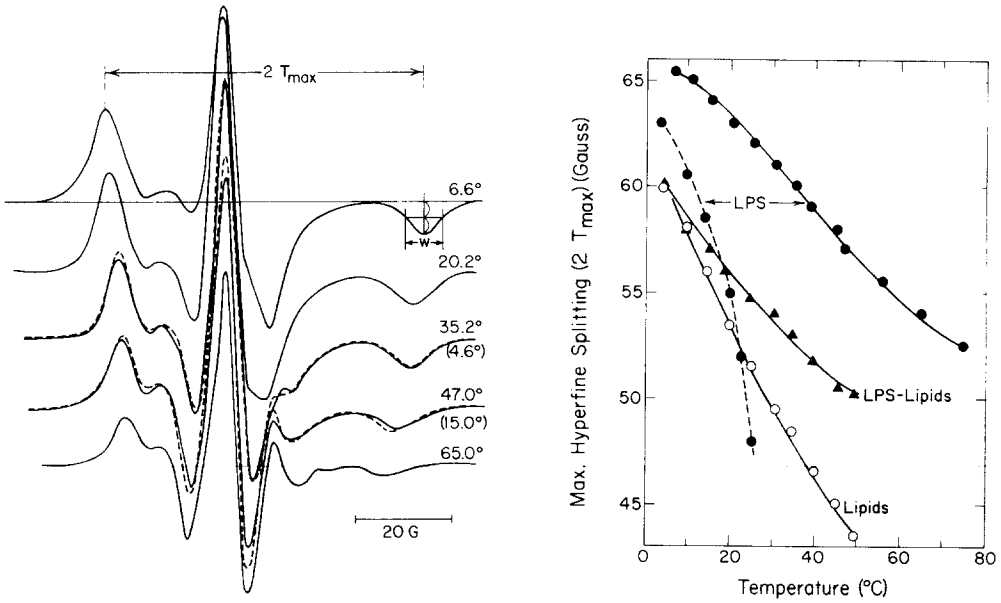


Fig. 4. ESR spectra of 5-nitroxide stearate incorporated into a dispersion of lipopolysaccharide. 5 Nitroxide stearate was added to lipopolysaccharide in 0.2 M NaCl/0.01 M $MgCl_2$ in a ratio of 0.9 mol per 100 mol of fatty acid residues in lipopolysaccharide. For comparison, spectra of 5-nitroxide stearate incorporated into lipid dispersions in 0.2 M NaCl/0.01 M $MgCl_2$ (0.4 mol per 100 mol of fatty acid residues in lipids) are shown in dotted lines.

Fig. 5. Temperature dependence of hyperfine splitting ($2T_{max}$) of spin-labeled fatty acids incorporated into model bilayers. 5-Nitroxide stearate (solid lines) was added to dispersions of lipopolysaccharide (●), lipids (○), and lipopolysaccharide-lipid mixture (▲) in 0.2 M NaCl/0.01 M $MgCl_2$ at the ratio of 0.9, 0.4, and 0.26 : 100 (mol of labeled fatty acids:mol of fatty acid residues in dispersions). 12-Nitroxide stearate (dotted lines) was added to lipopolysaccharide in 0.2 M NaCl/0.01 M $MgCl_2$ at a ratio of 0.4 : 100. The lipopolysaccharide-lipid mixture had been purified by equilibrium density gradient centrifugation as in Fig. 2, tube C, and contained lipopolysaccharide and phospholipids at a weight ratio of 0.7 : 1, or in such a way that the molar ratio of fatty acid residues in lipopolysaccharide and lipids was 0.44 : 1.

lently linked "backbone" structure. Thus the proximal portion of the fatty acid chains can never attain unhindered mobility, as in this system the contribution of molecular motion is presumably insignificant in comparison with intramolecular motion. This interpretation is supported by other lines of evidence. First, the hydrocarbon "fluidity" as measured by the solubility of TEMPO (Fig. 6) showed a continuous slow increase over the temperature range tested, and showed no sign of either a sharp rise (i.e. cooperative melting) or the presence of plateau (i.e. complete melting). Secondly, the melting profile obtained with 12-nitroxide stearate as the probe was very different from that obtained with 5-nitroxide stearate. The former label probes the mobility of the portion of the fatty acid chain near the methyl end, and is expected to be little affected by the anchoring at the carboxyl end. As expected, 12-nitroxide stearate indicated a rapid melting of the distal portions of the hydrocarbon chains in lipopolysaccharide (Fig. 5).

When the lipopolysaccharide-lipid complex was probed with 5-nitroxide stearate, the $2T_{max}$ values obtained were similar to those of phospholipids at low temperatures, but attained values much higher than the latter at tempera-

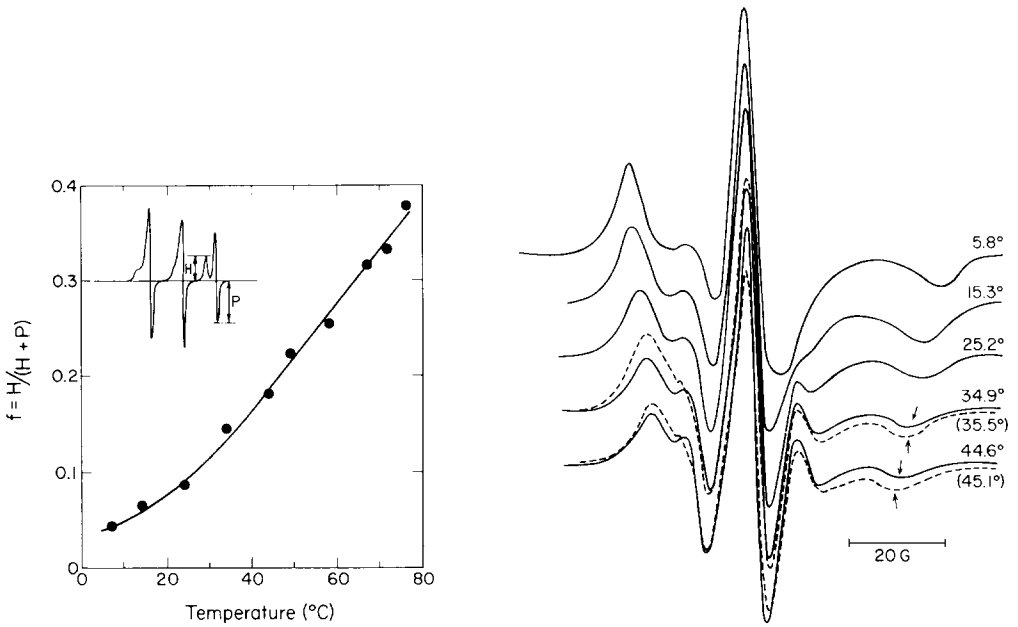


Fig. 6. Solubility of TEMPO in lipopolysaccharide dispersions. The dispersion containing, in 0.2 M NaCl/0.01 M $MgCl_2$, lipopolysaccharide (11 mg/ml) and TEMPO (5 mM), was centrifuged , and the pellet suspended in a small amount of the supernatant was used in the partition experiment. The index f was determined as shown in the figure.

Fig. 7. ESR spectra of 5-nitroxide stearate incorporated into membranes. 5-Nitroxide stearate was added to an outer membrane preparation at the ratio of 1.5 mol/100 mol of fatty acid residues in membrane phospholipids. For comparison, the spectra of 5-nitroxide stearate added to an inner membrane preparation (4.3 mol/100 mol of fatty acid residues in membrane phospholipids) are shown in dotted lines. Note that the high field throughs appear much wider in the inner membrane spectra (arrows).

tures higher than 20°C (Fig. 5). The spectra never showed any indication of major heterogeneity. For example, when the half-height width of the high field peak (w in Fig. 4) was measured as an index of heterogeneity in the environment of the probe, the values were 8.4—9.6 G for lipopolysaccharide, 8—10 G for lipid dispersions, and 8—9.6 G for lipopolysaccharide-lipid complex at temperatures ranging from 5 to 40°C. In contrast, the spectra of the mixtures of phospholipid vesicles and lipopolysaccharide, such as the sample 1 of Fig. 3 before centrifugation, were indicative of pronounced heterogeneity, and contributions of both phospholipids and lipopolysaccharide were clearly visible (not shown). These results suggest strongly that in the lipopolysaccharide-phospholipid complex prepared in 0.2 M NaCl/0.01 M $MgCl_2$, lipopolysaccharide and phospholipid molecules exist as homogeneous, finely interspersed bilayers and do not segregate into separate, large domains.

*ESR spectra of membranes*

The spectra of outer and inner membranes were determined with 5-nitroxide stearate and 12-nitroxide stearate as probes (Figs. 7 and 8). We note particularly the following points. (i) Spectral features were clearly defined and lines were quite sharp, in comparison with the spectra published by Rottem et al. [23],

who most probably used excessive amounts of spin labels. (ii) Outer and inner membranes were very similar in $2T_{max}$ values and in the shape of the spectra. (iii) $2T_{max}$ values of both membranes were intermediate between those of lipopolysaccharide and those of lipid dispersions. The thermal melting behavior of the membranes, judged from the slope of the $2T_{max}$ versus temperature plots, resembled that of the lipid dispersion, although the $2T_{max}$ values of the membranes were larger by 4—6 G at each temperature. (iv) Although there were some indications of heterogeneity in the environment of the probe, second peaks or shoulders corresponding to lipopolysaccharide were never observed in the outer membrane. In fact, the shape of the high field trough suggested that the environment in the outer membrane was actually less heterogeneous than that in the inner membrane (Fig. 7).

In the outer membrane, lipopolysaccharide and phospholipids may exist as interspersed, mixed bilayer (or monolayer) or form separate domains. In the latter case, spin-labeled stearic acids may distribute only in the phospholipid region or in both the phospholipid region and the domains occupied by lipopolysaccharide. The last possibility seems unlikely on the basis of the following argument. Motions of hydrocarbon chains of phospholipids is usually much

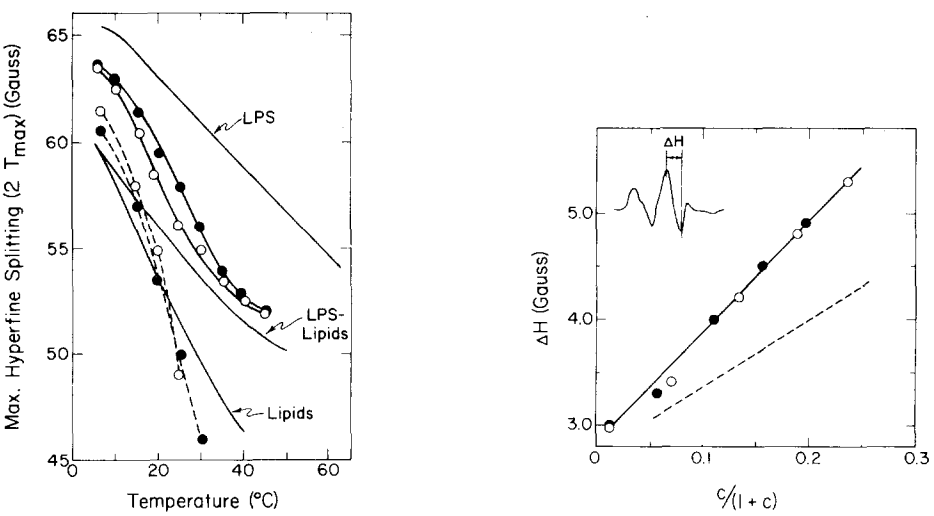


Fig. 8. Temperature dependence of the maximum hyperfine splitting ($2T_{max}$) of spin-labeled stearates in the outer and inner membranes. 5-Nitroxide stearate (solid lines) and 12-nitroxide stearate (dotted lines) were added to the outer membrane (•) at ratios of 1.5 and 1.9 : 100 (mol of labeled fatty acid : mol of fatty acid residues in membrane phospholipids). They were also added to the inner membrane (○) at ratios of 4.3 and 3.0 : 100. Solid lines without symbols are the $2T_{max}$ data obtained with model systems containing 5-nitroxide stearate; these were taken from Fig. 5.

Fig. 9. Exchange broadening of central line width. 5-Nitroxide stearate was added in varying amounts to samples of the outer membrane (38 μl, containing 0.398 μmol phospholipids) (•) and to those of the inner membrane (30 μl, containing 0.195 μmol phospholipids) (○), and ΔH values were determined from the expanded spectra obtained at 40°C. These values were plotted against $c/(1 + c)$, where $c$ is the molar ratio, 5-nitroxide stearate/phospholipids in the membrane [18]. Dotted line shows the results expected for the outer membrane in one of the following situations. (i) lipopolysaccharide and phospholipids form a homogeneous mixed phase in the membrane. (ii) Even if lipopolysaccharide and phospholipid occupy separate domains, 5-nitroxide stearate distributes evenly in both phases.

more restricted in biological membranes than in liposomes [24,25], and we can expect the same for hydrocarbon chains of lipopolysaccharide. Thus if the probes were distributed in the pure lipopolysaccharide domain, they should produce signals with $2T_{max}$ values larger than those obtained with lipopolysaccharide dispersions, but such signals were not found. We also believe that our observation does not support the occurrence of the homogeneous, mixed bilayer containing both lipopolysaccharides and lipids. We have seen, that in a model bilayer containing lipopolysaccharides and lipids at a ratio of 0.7 : 1 (w/w), the hydrocarbon chains were strongly immobilized especially at high temperatures, so that at 45°C the $2T_{max}$ values were increased by about 6 G over those in lipid dispersions (Fig. 5). The outer membrane contains even more lipopolysaccharides, and the lipopolysaccharide-to-lipid ratio here reaches 1 to 1.5 (w/w) [9,21]. Thus if most phospholipids in the outer membrane existed in lipopolysaccharide-lipid mixed bilayers, the probes in the outer membrane should appear more and more immobilized at higher temperature, in comparison to those in the inner membrane that contains phospholipid-only bilayers. The $2T_{max}$ values, however, were actually identical in outer and inner membranes at 45°C. The slight difference (about 2 G) at 20—25°C can be easily explained by the higher relative content of phosphatidylethanolamine in the outer membrane [21], and/or by different degrees of immobilization by membrane proteins. These results suggested that phospholipids and lipopolysaccharides in the outer membrane do not exist as a mixed, homogeneous phase, but rather occupy separate domains and that 5-nitroxide stearate enters only into the phospholipid domains in the temperature range studied. We could not rule out, however, an alternative, although unlikely, possibility that the immobilizing effect of lipopolysaccharide on phospholipid hydrocarbon chains was cancelled out by a weaker immobilizing effect of outer membrane proteins. Thus a confirmation from a different approach was necessary, and we have accordingly carried out the experiments described in the next section.

*Exchange broadening of line width*

At high concentrations of spin label, label molecules in membranes collide with each other and produce broadening of line width in ESR spectra. The extent of broadening depends on collision frequency, which in turn depends both on the fluidity of the membrane, which determines the diffusion rate, and on the concentration of the label. Since the outer and inner membranes show essentially identical $2T_{max}$ values for 5-nitroxide stearate at 40°C, we can assume that the fluidity of the environment of the probe is probably very similar. Thus by determining line broadening at various overall concentrations of 5-nitroxide stearate in these two membranes, we can determine the actual concentration of 5-nitroxide stearate in the domains it is distributed in, and from this we can assess the relative size of the domains.

The width from the top of the central peak to its bottom on the high field side (see Fig. 9) was measured at various concentrations of 5-nitroxide stearate. Preliminary experiments showed that 5-nitroxide stearate was so effectively incorporated into the membranes that much error was not introduced by disregarding the very small portion of unincorporated 5-nitroxide stearate. Thus if 5-nitroxide stearate is distributed only in phospholipid mono- and

bilayer regions in the membrane, line broadening should be proportional to $c/(1 + c)$, where $c$ is mole of 5-nitroxide stearate added/mol of phospholipids present in the membrane [18]. As seen in Fig. 9, results on outer and inner membranes fit into a single straight line. If phospholipids and lipopolysaccharides exist as a mixed mono- or bilayer in the outer membrane, the effective concentration of 5-nitroxide stearate in this domain would be about one-half of $c$ in Fig. 9, as there are about as many fatty acid chains in lipopolysaccharide as those present in phospholipids [9]. This situation will produce results expressed as a dotted line (Fig. 9), which clearly does not fit the data obtained with the outer membrane. These results thus suggest that phospholipid and lipopolysaccharide form separate domains in the outer membrane, and that spin-labeled stearic acid probes apparently enter only into the phospholipid domains.

## Discussion

In this work we studies the structure of the outer membrane of *S. typhimurium* by adding spin-labeled fatty acids to the outer membrane as well as to the inner membrane and to various model bilayers. We found that the ESR spectra of the outer membrane were remarkably similar to those of the inner membrane in spite of the presence of a large amount of lipopolysaccharide in the former, and comparison with the behavior of a model lipopolysaccharide-phospholipid bilayer (Fig. 8), as well as the exchange broadening experiments (Fig. 9), enabled us to establish that most of the phospholipid molecules in the outer membrane occupied domain(s) separate from that occupied by the lipopolysaccharide molecules, and that spin-labeled stearic acid probes partition apparently only into the phospholipid domains. These phospholipid domains are presumably rather large, or are not juxtaposed to the lipopolysaccharide domains, as the thermal melting profile of the former (Fig. 8) was very similar to that of the inner membrane, and showed little influence of the presence of lipopolysaccharide.

The segregation of lipopolysaccharides and phospholipids in discrete domains in the outer membrane, even near the temperature of growth (cf. Fig. 9), is obviously inconsistent with the older models of outer membrane, which assumed the presence of lipopolysaccharide-phospholipid mixed bilayer as the basic continuum of the membrane. On the other hand, these results are fully consistent with the more recent results [9] with non-penetrable, covalent labeling reagents, suggesting that most phospholipid molecules are located in the inner leaflet in the outer membrane, as well as the results showing that most lipopolysaccharide molecules are located in the outer half of the membrane [8].

A survey of the literature shows that published results are consistent with the segregation of lipopolysaccharide and phospholipid domains in the outer membrane. Thus Overath et al. [26] found, with fluorescent probes and by X-ray diffraction, that hydrocarbon chains of phospholipids in the outer membrane "melt", or become disordered, at the same temperature as those in the cytoplasmic membrane. In view of our observations on the melting behavior of lipopolysaccharide and lipopolysaccharide-phospholipid mixed bilayer, these

results clearly indicate that a large fraction of phospholipids in the outer membrane exists as a pure mono- or bilayer of phospholipids, without entering into association with lipopolysaccharides. Overath et al. also found that a considerable fraction of the phospholipids in the outer membrane does not take part in the cooperative melting, presumably because it interacts strongly with proteins [26]. This is also consistent with the following observation of ours. In the inner membrane, the probe is clearly in a heterogeneous environment at 30—45°C (see the high field troughs of dotted curves in Fig. 7); this situation presumably reflects the presence of "free" phospholipids with smaller hyperfine splitting as well as "protein-immobilized" phospholipids with larger $2T_{max}$ values. The spectra of the outer membrane in the same temperature range appear more homogeneous (Fig. 7), presumably because the relative contribution of the "immobilized" signal is greater.

Spin label probes were previously used by Rottem et al. [23] in their study of the structure of *Proteus mirabilis* outer membrane. They obtained results that are generally similar to ours, but they could not choose between various possible structural models, as they did not study the model bilayers. One difference between their results and ours is that they found somewhat larger difference in the mobility of probes between the outer and inner membranes (1—5 G in $2T_{max}$ values). Possible reasons for this discrepancy include the difference in bacterial strains, as well as perturbations caused by the use of excessive amounts of spin labels by Rottem et al. [23] (see Results).

Since lipopolysaccharide molecules are segregated away from phospholipids in the outer membrane, the hydrocarbon chains of lipopolysaccharide must be in a very viscous environment, in view of the results on lipopolysaccharide bilayers (Fig. 5). This conclusion is obviously consistent with the rates of lateral diffusion of lipopolysaccharide, which were almost 10 000 times slower than the rates of diffusion of phospholipids [27].

Although our data indicate that most phospholipids in the outer membrane exist as phospholipid mono- or bilayer, the line broadening experiments were not precise enough to exclude the possibility that a minor fraction of phospholipids exist in association with lipopolysaccharides. Even if this were the case, only a very small fraction, by weight, of such "mixed bilayer" regions could be occupied by phospholipids, since the total weight of phospholipids in the outer membrane of Rc mutants is only 65—94% [11,21] of that of lipopolysaccharides. We can thus conclude that at least the lipopolysaccharide-phospholipid mixed bilayers of the type that had previously been studied, i.e. those with the phospholipid/lipopolysaccharide ratio (w/w) of 3 or more [16], cannot serve as models of the outer membrane. Such a structure would rather resemble the situation in the inner membrane, where lipopolysaccharide molecules would be anchored to the membrane presumably by insertion into the phospholipid bilayer structure, while the saccharide chain of lipopolysaccharide gets elongated [1]. Once synthesized, the lipopolysaccharide and phospholipid molecules would get incorporated into the outer membrane in a completely asymmetric manner; the mechanism of this process remains a topic for future study.

The thermal melting behavior of lipopolysaccharide was quite different from that of phospholipids (Fig. 5). Results with 12-nitroxide stearate suggested that the portion of the hydrocarbon chain near the methyl end became disordered

rapidly as the temperature increased, but the more proximal sections, probed by 5-nitroxide stearate, did not show any precipitous decrease of viscosity in the temperature range studied, and retained considerable order even at 75°C. Partition experiments with TEMPO as the probe also corroborated these conclusions. This behavior is presumably due to the fact that up to 18 fatty acid chains are covalently connected to a single backbone structure. Such a highly ordered structure of hydrocarbon chains would suggest that the main function of lipopolysaccharide in the outer membrane is structural, and differs much from that of phospholipids which perform the precise adjustment of membrane fluidity for the more dynamic functions of the membrane. Indeed the fatty acid composition of lipopolysaccharide of *Salmonella* was found to be constant, regardless of the temperature at which the organism was grown (Gmeiner, J. and Nikaido, H.,, unpublished results). Furthermore, it is possible that the more ordered membrane interior, produced by the presence of hydrocarbon chains of lipopolysaccharides, is important in restricting the passage of hydrophobic molecules across the outer membrane (see ref. 28).

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