Biochimica et Biophysica Acta, 774 (1984) 181–187
Elsevier

BBA 72149

A PROTON AND DEUTERIUM NUCLEAR MAGNETIC RESONANCE STUDY OF ORIENTATIONAL ORDER IN AQUEOUS DISPERSIONS OF LIPOPOLYSACCHARIDE AND LIPOPOLYSACCHARIDE/DIPALMITOYLPHOSPHATIDYLCHOLINE MIXTURES

ALEX L. MACKAY [a], CHRISTINE P. NICHOL [b,*], GERALD WEEKS [b] and JAMES H. DAVIS [c]

Departments of Physics [a] and Microbiology [b], University of British Columbia, Vancouver, B.C. and [c] Biophysics Interdisciplinary Group, Department of Physics, University of Guelph, Guelph, Ontario (Canada)

(Received January 4th, 1984)

Key words: Lipopolysaccharide; Phospholipid; Phosphatidylcholine; Orientational order; ^2H-NMR

Magnetic resonance measurements of orientational order have been carried out in aqueous dispersions of lipopolysaccharide derived from *Escherichia coli* K-12 cells and the phospholipid dipalmitoylphosphatidylcholine. Proton magnetic resonance experiments demonstrated that the orientational order of pure lipopolysaccharide dispersions at 6°C was one-third of that predicted for completely rigid lipopolysaccharide molecules. The orientational order decreased by a factor of 2 as the temperature was increased to 40°C. The lipopolysaccharide orientational order was not affected significantly by the presence of phospholipid in the dispersion. Deuteron magnetic resonance experiments indicated that the dispersions of phospholipid and lipopolysaccharide are homogeneously mixed and that the presence of lipopolysaccharide decreased the phospholipid average hydrocarbon chain order in the gel phase and increased it in the liquid-crystalline phase. In the temperature range between 26 and 40°C, there was a coexistence of gel and liquid-crystalline phospholipid.

Introduction

A number of studies of molecular motion in Gram-negative bacteria [1–6] have indicated that bacterial outer membranes are more rigid than bacterial cytoplasmic membranes. In particular, recent ^{2}H-NMR investigations [3,4] of *Escherichia coli* (L-51 strain) have demonstrated that the phospholipid hydrocarbon chain orientational order of the cytoplasmic membrane is significantly smaller than that of the outer membrane throughout a large temperature range. Since a major difference between the *E. coli* cytoplasmic and outer membranes is the presence of the molecule lipopolysaccharide in the outer membrane [7], it is appropriate to ask whether the additional rigidity of the outer membrane is due to the presence of the lipopolysaccharide.

The lipopolysaccharide molecule contains a hydrophobic region called lipid A and a hydrophilic polysaccharide chain that varies in length between bacterial species and strains. The lipopolysaccharide from the rough *E. coli* strain K-12 has a relatively short polysaccharide chain [7,10]. It exists in bilayer structures and undergoes a thermal transition when in aqueous dispersions or in the anhydrous form [1,11–16]. Aqueous dispersions of phospholipids also form bilayers in the presence of lipopolysaccharide [11,13,14] and exhibit a hydrocarbon chain thermal phase transition [11,13,14, 17]. Phospholipid hydrocarbon chain order and domain formation in lipopolysaccharide/

* Present address: Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, B.C., Canada.

Abbreviation: DPPC, dipalmitoylphosphatidylcholine.

phospholipid dispersions [18,19] and *E. coli* outer and cytoplasmic membrane preparations [1] have been studied by electron paramagnetic resonance spin-label experiments.

We have prepared aqueous dispersions of *E. coli* lipopolysaccharide (strain K-12) and *E. coli* lipopolysaccharide (strain K-12)/dipalmitoylphosphatidylcholine (DPPC) mixtures in order to investigate the effect of lipopolysaccharides on the physical state of DPPC model membranes by ^{1}H- and ^{2}H-NMR. The phospholipid DPPC was chosen because its physical state in aqueous dispersions has been studied extensively [8,9].

Materials and Methods

Materials. L-α-Phosphatidylcholine, β-γ-di-d_{31}-palmitoyl (98% deuterated) was obtained from Lipid Specialties Inc., Boston. L-α-Phosphatidylcholine, βγ-dipalmitoyl was obtained from Calbiochem. *E. coli* (strain K-12) lyophilized cells ATCC 1078 B grade were also obtained from Calbiochem. ^2H_2O (99.8% ^{2}H) was obtained from Merck, Sharpe and Dohme, Canada, Ltd.

Isolation of lipopolysaccharides. The lipopolysaccharide was extracted from 50 g of *E. coli* by phenol/chloroform/petroleum ether extraction and precipitated with water [20]. The dry lipopolysacchride (130 mg) was resuspended in water and centrifuged at 45000 rpm in a Beckman 60 Ti rotor for 4 h at 5 °C. The pellet was resuspended in water and stored at −20 °C. The ultraviolet spectra showed that there was no protein or ribonucleic acid present in the preparation.

Preparation of the lipopolysaccharide/DPPC dispersions. The two lipopolysaccharide/DPPC liposome samples were prepared by the method used by Nikaido et al. [18]. 10 mg lipopolysaccharide was dissolved in 0.75 ml 0.01 M $MgCl_2$, 0.14 M NaCl in ^2H_2O and added to a dry film of DPPC (10 mg) in a screw-cap tube. The tube was placed in a Megason bath and the mixture was sonicated for 13 min at 45 °C. The suspension was diluted 4-fold with ^2H_2O, sonicated with a Bronwill Biosonik 0.5-inch probe for 3.5 min at 45 °C, dried under a stream of N_2 and redispersed in the original volume of ^2H_2O by sonicating in the Megason bath at 45 °C for 10 min. All sonications were performed under N_2.

The predeuterated DPPC/lipopolysaccharide liposome sample was prepared as above, except that five-times more material and H_2O were used throughout. After sonication with the 0.5-inch probe, the 20-ml suspension was concentrated to 4 ml by ultrafiltration on an XM 100 Al Amicon filter under N_2 at 20 lb/inch^2. The lipopolysaccharide/DPPC-d_{62} mixture was then dried down at 45 °C under N_2 and dispersed in 1 ml H_2O by sonication in the Megason bath at 45 °C.

Characterization of the lipopolysaccharide/DPPC preparations. Electron microscopy of lipopolysaccharide/DPPC samples, negatively stained with 2% ammonium molybdate showed a variety of structures present, from 300 Å vesicles to 5000 Å vesicles and various multilamellar shapes. The majority of the sample was in the larger structures, as is clearly evident in the NMR spectra.

To establish that the lipopolysaccharide and the DPPC were in a homogeneous mixture [21], a portion of each sample used for NMR was layered on a 10–30% sucrose gradient containing 0.14 M NaCl and 0.01 M $MgCl_2$ on a cushion of 60% sucrose. The gradients were centrifuged at 35000 rpm for 20 h at 4 °C using a Beckman SW 50 rotor. After visual observation, the gradient was divided into four 1-ml fractions at the top of the tube and two 0.5-ml sections at the bottom. For each portion, the DPPC content was estimated by phosphate analysis [22] and the lipopolysaccharide content was determined by Osborne's modification [23] of the 2-keto-3-deoxyoctanate estimation [24]. From standard solutions ketodeoxyoctanate was estimate to be 12% of the K-12 lipopolysaccharide.

For the ^{1}H-NMR preparation (10 mg lipopolysaccharide and 10 mg DPPC), a single band which contained all of the lipopolysaccharide and 80% of the DPPC was observed in the gradient at density ρ between 1.08 and 1.12. For the ^{2}H-NMR preparation (50 mg lipopolysaccharide and 50 mg DPPC-d_{62}), a single band which contained 95% of the lipopolysaccharide and 86% of the DPPC-d_{62} was obtained near the interface between the gradient and the 60% sucrose.

Proton NMR. The ^{1}H-NMR results were acquired at 90 MHz with a Bruker SXP 4-100 NMR spectrometer. For each free induction decay, 1000 scans were accumulated with a recovery time of 1 s. Temperature control of the sample was

achieved with an air-flow device, to an accuracy of 0.5 °C although a temperature gradient of about 1 °C existed across the samples.

Quadrature free induction decay data after a 90 ° pulse were collected on resonance with a 2 μs dwell time in 2048 data points. Since the first 8 μs of the free induction decay was unobtainable due to receiver recovery from the pulse, the out-of-phase free induction decay was extrapolated to $t = 0$ by means of the function $S(t) = S(0) \cdot (1 - M_2 t^2/2)$ and the in-phase free induction decay was extrapolated using $S(t) = S(0) M_1 t$ [8]. The extrapolated free induction decay data were Fourier-transformed and second moments were calculated from the spectra by integration [8]. The error in the M_2 measurement was estimated to be $\pm 5\%$.

Deuteron NMR. ^{2}H-NMR spectra were obtained at 37.18 MHz employing the quadrupolar echo technique (90_0-τ-90_{90}) using 90 ° pulses of length 4.5 μs separated by a τ value of 60 μs. Typically, 10000–15000 scans were accumulated at a repetition rate of two scans/s. The sample temperature was maintained by an oven so that the temperature gradient across the sample was typically much less than 1 deg. C. The sample was allowed at least 30 min to come to equilibrium following each successive 1 or 2 deg. C temperature increment.

Results and Discussion

Proton magnetic resonance

Proton NMR spectra were acquired from lipopolysaccharide/^2H_2O, lipopolysaccharide/DPPC-d_{62}/^2H_2O and lipopolysaccharide/DPPC/^2H_2O samples as a function of temperature between 6 and 45 °C. These spectra, which are dominated by the dipolar interactions between proton pairs, are rather broad and they contain no resolved splittings or chemical shifts. The spectral moments have been found to be appropriate parameters for the characterization of such spectra [8]. It sould be emphasized that very high fidelity NMR spectra are essential for accurate moment measurements. The residual second moments M_{2r} of the ^{1}H-NMR spectra for each sample are plotted as a function of temperature in Fig. 1.

The rigid lattice M_2 for lipopolysaccharide (i.e., the second moment one would measure in the absence of motion) was estimated to be about $1.4 \cdot 10^{10}$ s^{-2} using a tentative structure of *E. coli* K-12-derived lipopolysaccharide (see Fig. 5 in Ref. 7). At 6 °C, the residual second moment, M_{2r}, for lipopolysaccharide in water is $4.0 \cdot 10^9$ s^{-2}, indicating the presence of considerable motion of the lipopolysaccharide on the NMR time scale ($\tau M_2^{-1/2} \simeq 10^{-5}$ s). This reduction in the lipopolysaccharide M_{2r} from its rigid lattice value is similar to that observed for DPPC/^2H_2O dispersions in the gel phase [8].

The M_{2r} for lipopolysaccharide decreases by about $2.5 \cdot 10^9$ s^{-2} in the temperature range

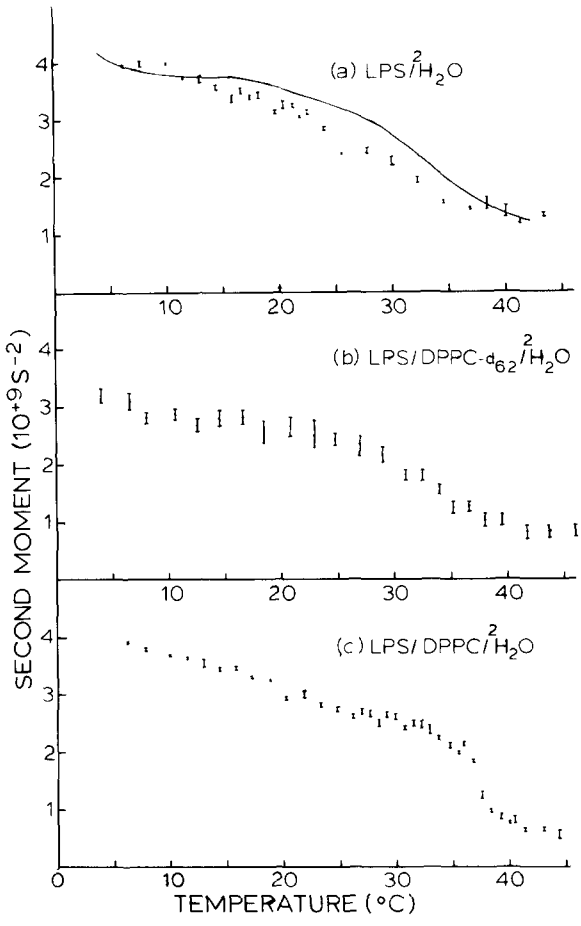

Fig. 1. ^{1}H-NMR second moments as a function of temperature for the dispersions of (a) lipopolysaccharide/^2H_2O, (b) lipopolysaccharide/DPPC-d_{62}/^2H_2O and (c) lipopolysaccharide/DPPC/^2H_2O. In (a) the continuous line represents M_2[lipopolysaccharide] from the lipopolysaccharide/DPPC-d_{62}/^2H_2O sample.

10–35 °C. This drop in M_{2r} can be accounted for by a change in physical state of the fatty acyl chains of the lipopolysaccharide similar to that experienced by phospholipid acyl chains at their gel to liquid-crystalline transition [8]. A variety of other physical techniques including electron paramagnetic resonance [1,15], differential scanning calorimetry [16], light scattering [16], freeze-fracture electron microscopy [13], X-ray diffraction [11,12,14] and fluorescent probe intensity measurements [11] have indicated a wide lipopolysaccharide hydrocarbon chain phase transition between 5 and 25 °C. The temperature range over which this transition has been observed varies considerably and depends upon the source of lipopolysaccharide and on the measurement technique used.

Lipopolysaccharide / DPPC-d_{62} / ^2H_2O

For the lipopolysaccharide/DPPC-d_{62}/^2H_2O preparation, the ^1H M_{2r} (see Fig. 2b) has contributions from protons on the lipopolysaccharide and from the DPPC headgroup (and a small contribution from residual ^1H on the hydrocarbon chain). If one assumes that the M_{2r} for the headgroup is similar to that from pure DPPC-d_{62} dispersions [8], the contribution of the lipopolysaccharide to the M_{2r} for the whole sample can be obtained using the relation:

$$M_{2r} = \left\{ \begin{array}{l} \text{Fraction of } ^1\text{H } M_2\text{[lipopolysaccharide]} \\ \text{in lipopolysaccharide} \end{array} \right\}$$

$$+ \left\{ \begin{array}{l} \text{Fraction of } ^1\text{H } M_2\text{ [DPPC-}d_{62}\text{]} \\ \text{in DPPC-}d_{62} \end{array} \right\}$$

These fractions were derived with the assumption that the lipopolysaccharide contains 250 nonexchangeable protons per molecule, while DPPC-d_{62} contains 18. The continuous line in Fig. 1a corresponds to the M_2[lipopolysaccharide] values for the lipopolysaccharide/DPPC-d_{62}/^2H_2O sample calculated from the above equation. The two curves are similar, indicating that the orientational order of the lipopolysaccharide is not affected substantially by the presence of phospholipid in the bilayer. In a ^{31}P-NMR study of liposomes containing native lipopolysaccharides from *E. coli* K-12 and total phospholipids from *E. coli* [13], it was concluded that the orientational order of the lipopolysaccharide at 37 °C was decreased by the presence of phospholipid. This apparent discrepancy may be because the *E. coli* K-12 phospholipids have unsaturated acyl chains. It should be emphasized here that we have established conclusively from sucrose-gradient measurements that our liposomes contain both lipopolysaccharides and DPPC.

Lipopolysaccharide / DPPC / ^2H_2O

The values for M_{2r} (see Fig. 1c) of the lipopolysaccharide/DPPC/^2H_2O sample decrease considerably between 6 and 45 °C. The gel to liquid-crystalline phase transition of the DPPC hydrocarbon chains, which is identified with the more rapid decrease of M_{2r} between 35 and 40 °C, is broader and is displaced to a lower temperature.

^2H-NMR of lipopolysaccharide / DPPC-d_{62} / H_2O dispersions

At 38 °C and above, the ^{2}H-NMR spectra of

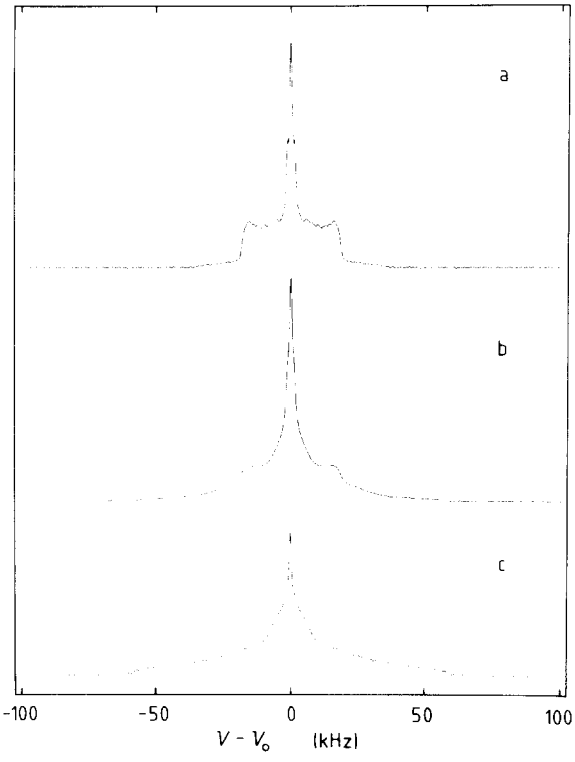

Fig. 2. ^{2}H-NMR spectra from an lipopolysaccharide/DPPC-d_{62}/^1H_2O dispersion in (a) the liquid-crystalline phase at 40 °C, (b) a region of coexistence of liquid-crystalline and gel phases at 36 °C and (c) the gel phase at 22 °C.

(chain perdeuterated) lipopolysaccharide/DPPC-d_{62}/H_2O dispersions are characteristic of the lamellar liquid-crystalline phase [25] as illustrated by the spectrum at 40 °C in Fig. 2a. At 38 °C and below, the system enters a two-phase region with both liquid crystalline and gel components. This coexistance of the two phases is clearly demonstrated by the spectrum at 36 °C (Fig. 2b) where the sharp edges of the liquid-crystalline-phase spectrum are clearly seen on top of the much broader gel-phase spectrum. Eventually, the transformation from liquid-crystalline to gel phase is complete so that at 22 °C (Fig. 2c), the spectrum is characteristic of the gel phase. At all temperatures, there is a small isotropic peak at the center of the spectrum. This peak is at least partially due to natural abundance of ^2H in the water but may also contain contributions from small phospholipid/lipopolysaccharide particles in the sample. Since the intensity of this isotropic peak is only a few percent of the total integrated intensity, most of the sample consists of large structures where isotropic averaging does not occur on the ^2H-NMR timescale (no greater than 10^{-5} s).

The comparison, in the liquid-crystalline phase, of the lipopolysaccharide/DPPC-d_{62}/H_2O system with pure DPPC-d_{62}/H_2O system is facilitated by 'de-pakeing' the powder pattern spectrum to obtain the spectrum characteristic of an oriented sample, i.e., a sample where all of the bilayers have the same orientation with respect to the magnetic field [26]. The 'de-Paked' spectrum at 45 °C (Fig. 3b) is similar to that of DPPC-d_{62}/H_2O at 45 °C (Fig. 3a), the most significant difference being the shift of the largest peak to a higher frequency for the sample containing lipopolysaccharides. The distribution of quadrupolar splittings, given explicitly in the de-paked spectrum, has the same overall shape for the two systems, but the average quadrupolar splitting is substantially increased by the presence of lipopolysaccharides in the bilayer.

The moments of the ^2H-NMR spectrum are sensitive to changes in the degree of orientational order. The first moment, M_1, which for systems with axial symmetry is proportional to the average quadrupolar splitting, is plotted versus temperature in Fig. 4a. The large increase in the value of M_1 on cooling from 40 to 26 °C is due to the conversion from the liquid-crystalline to the gel phase. Below 25 °C, the value of M_1 slowly increases as the temperature is lowered due to a

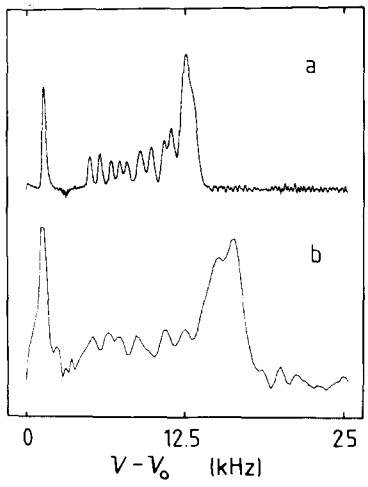

Fig. 3. The 'de-Paked' spectrum for (a) a pure DPPC-d_{62}/^1H_2O dispersion at 45 °C and (b) an lipopolysaccharide/DPPC-d_{62}/^1H_2O dispersion at 45 °C.

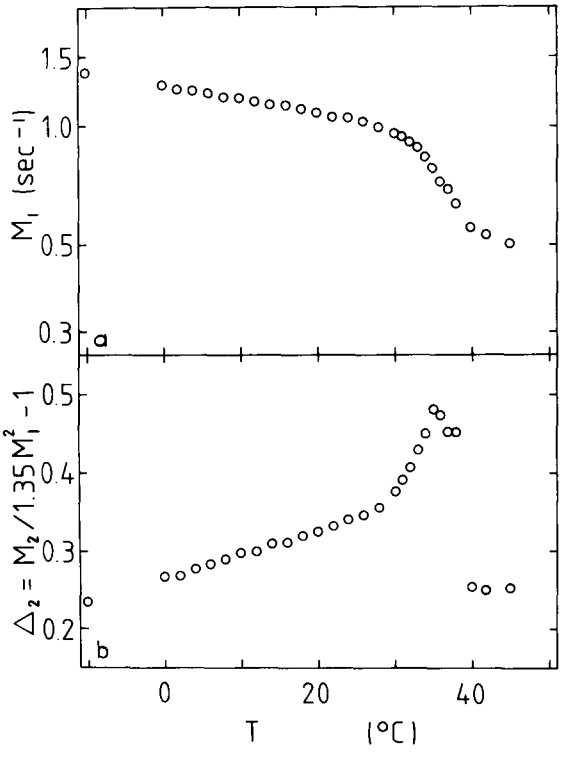

Fig. 4. The ^2H-NMR first moment, M_1, (a), and the fractional mean square width of the distribution of quadrupolar splittings, Δ_2, (b), for the lipopolysaccharide/DPPC-d_{62}/^1H_2O dispersion as a function of temperature.

gradual decrease in molecular motion and the values of M_1 for lipopolysaccharide/DPPC-d_{62}/H_2O are consistently lower than those of the pure DPPC-d_{62}/H_2O system.

As indicated by the de-Paked spectra, in the liquid-crystalline phase the magnitude of M_1 is significantly larger in the lipopolysaccharide/DPPC-d_{62}/H_2O system than in DPPC-d_{62}/H_2O, e.g., at 45 °C, M_1 (lipopolysaccharide/DPPC) = $5.31 \cdot 10^4$ s^{-1} while M_1 (DPPC) = $4.60 \cdot 10^4$ s^{-1}. The higher moments of the spectrum (M_2, etc) are also sensitive to the orientational order and can be used to extract additional information. The parameter $\Delta_2 = ((M_2/1.35\ M_1^2) - 1)$ which for systems of axial symmetry gives the fractional mean squared width of the distribution of quadrupolar splittings, is plotted versus temperature in Fig. 4b. The magnitude of Δ_2 above 40 °C in lipopolysaccharide/DPPC-d_{62}/H_2O is nearly twice that observed in DPPC-d_{62}/H_2O. In the liquid-crystalline phase, both the average quadrupolar splitting, given by M_1, and the width of the distribution of splittings, given by Δ_2, are dramatically increased by lipopolysaccharides.

In the two-phase region below 40 °C, the value of Δ_2 increases drastically as observed in other systems [9,4]. The large values observed between 26 and 38 °C are due to the coexistence of gel and liquid-crystalline phase phospholipids which have ^{2}H-NMR spectra with widely different splittings. The gradual decrease in Δ_2 below 26 °C is due to the rather subtle changes in molecular motion which occur in the gel phase [9].

Concluding remarks

The primary conclusions which can be drawn from these studies on lipopolysaccharide/DPPC/water dispersions are that: (i) the orientational order of the lipopolysaccharide molecules at 6 °C is about one-third that one would measure for rigid lipopolysaccharide molecules, it decreases by a factor of 2 as the temperature is increased to 40 °C and is not significantly different from that in pure lipopolysaccharide/water dispersions, (ii) in the liquid-crystalline phase, lipopolysaccharides and DPPC are randomly mixed, i.e., there is no evidence for persistence on the ^{2}H-NMR time-scale of domains rich in lipopolysaccharides (iii) the presence of lipopolysaccharides increases the average phospholipid hydrocarbon chain order in the liquid-crystalline phase and decreases it in the gel phase, and (iv) lipopolysaccharide/DPPC dispersions go through a broad two-phase region as the temperature is lowered below the transition temperature of pure DPPC, thus indicating that the lipopolysaccharide partitions into the liquid-crystalline phase.

The conclusion of some previous EPR studies on lipopolysaccharide/phospholipid dispersions suggesting the existence of domains rich in lipopolysaccharides [18,19], is not supported by our results.

In closing, it should be pointed out that this work supports the proposition that the greater rigidity, or higher order, of the outer membrane of *E. coli* compared to that of the cytoplasmic membrane [1–6] is due to the presence of lipopolysaccharides in the outer membrane.

References

1 Coughlin, R.T., Haug, A. and McGroarty, E.J. (1983) Biochim. Biophys. Acta 729, 161–166
2 Gally, H.-U., Pluschke, G., Overath, P. and Seelig, J. (1980) Biochemistry 19, 1638–1643
3 Davis, J.H., Nichol, C.P., Weeks, G. and Bloom, M. (1979) Biochemistry 18, 2103–2111
4 Nichol, C.P., Davis, J.H., Weeks, G. and Bloom, M. (1980) Biochemistry 19, 451–457
5 Janoff, A.S., Haug, A. and McGroarty, E.J. (1979) Biochim. Biophys. Acta 555, 56–66
6 Rottem, S. and Leive, L. (1977) J. Biol. Chem. 252, 2077–2081
7 Lugtenberg, B. and Van Alphen, L. (1983) Biochim. Biophys. Acta 737, 51–115
8 MacKay, A.L. (1981) Biophys. J. 35, 301–314
9 Davis, J.H. (1979) Biophys. J. 27, 339–358
10 Lugtenberg, B. (1981) Trends. Biochem. Sci. 6, 262–265
11 Emmerling, G., Henning, U. and Gulik-Krzywicki, T. (1977) Eur. J. Biochem. 78, 503–509
12 Burge, R.E. and Draper, J.C. (1967) J. Mol. Biol. 28, 205–210
13 Van Alphen, L., Verkleij, A., Burnell, E. and Lugtenberg, B. (1980) Biochim. Biophys. Acta 597, 502–517
14 Nakayama, H., Mitsui, T., Nishihara, M. and Kito, M. (1980) Biochim. Biophys. Acta 601, 1–10
15 Coughlin, R.T., Haug, A. and McGroarty, E.J. (1983) Biochemistry 22, 2007–2013
16 Van Alphen, L., Lugtenberg, B., Reitschel, E.T. and Mombers, C. (1979) Eur. J. Biochem. 101, 571–579
17 Liu, M., Onji, T., Snelgrove, N.E. (1982) Biochim. Biophys. Acta 710, 248–251

18 Nikaido, H., Takeuchi, Y., Ohnishi, S. and Nakae, T. (1977) Biochim. Biophys. Acta 465, 152–164
19 Takeuchi, Y. and Nikaido, H. (1981) Biochemistry 20, 523–529
20 Galanos, C., Luderitz, O. and Westphal, O. (1969) Eur. J. Biochem. 9, 245–249
21 Weiser, M.M. and Rothfield, L. (1968) J. Biol. Chem. 243, 1320–1328
22 Ames, B.N. (1966) Methods Enzymol. 8, 115–118
23 Osborne, M.J. (1963) Proc. Natl. Acad. Sci. USA 50, 499–506
24 Weissbach, A. and Hurwitz, J. (1959) J. Biol. Chem. 234, 705–709
25 Davis, J.H. (1983) Biochim. Biophys. Acta 737, 117–171
26 Bloom, M., Davis, J.H. and MacKay, A.L. (1981) Chem. Phys. Lett. 80, 198–202