Structure of *Escherichia coli* Membranes. Fatty Acyl Chain Order Parameters of Inner and Outer Membranes and Derived Liposomes†

Hans Ulrich Gally, Gerd Pluschke, Peter Overath, and Joachim Seelig*

ABSTRACT: *Escherichia coli* fatty acid auxotrophs (strain T2 GP) have been grown on media supplemented with elaidic-*9,10-d*$_2$ acid, replacing 80–90% of the natural fatty acids by this analogue. *E. coli* T2 GP strain is deficient in the synthesis of cardiolipin, and the cell membranes contain phosphatidylethanolamine (~80%) and phosphatidylglycerol (~20%) as the only phospholipid species. The phospholipid organization in cells, inner and outer membranes, and liposomes prepared from either membrane lipids or synthetic lipids (1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine) has been studied with deuterium and phosphorus-31 magnetic resonance and differential thermal analysis. The fatty acyl chains in elaidate-*9,10-d*$_2$-enriched *E. coli* inner and outer membranes are found to be less ordered by 10–20% than in corresponding model systems. Similar results are obtained for *E. coli* cells grown in the presence of oleic-*9,10-d*$_2$ acid and oleic-*12,12-d*$_2$ acid. Mg^{2+} ions induce the formation of quasi-crystalline Mg^{2+}–phosphatidylglycerol clusters in phosphatidylethanolamine–phosphatidylglycerol mixtures but have no effect on either pure phosphatidylethanolamine or intact inner or outer cell membranes. Synthetic 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine exhibits a lamellar to hexagonal liquid-crystal phase transition between 40 and 60 °C. The head group structure is not altered by this phase transformation whereas the average fatty acyl chain conformation becomes more disordered in the hexagonal phase. The lamellar → hexagonal phase transition is absent in phosphatidylethanolamine with a heterogeneous fatty acyl composition. It is also not detected in intact membranes of *E. coli* T2 GP cells.

The general morphology of most biomembranes is determined by a fluid lipid bilayer. A vast assortment of proteins is embedded in this lipid matrix, regulating the proper functioning of the membrane. A major issue at present is the question as to the extent to which a membranous protein perturbs its lipid environment and the significance this may have on biomembrane functioning [cf. Chapman et al. (1979) for a review].

By use of deuterium magnetic resonance as a physical tool, two approaches can be employed to investigate the problem of lipid–protein interactions in biomembranes. One possibility is the purification and delipidation of membrane-bound proteins followed by membrane reconstitution with selectively deuterated phospholipids (Dahlquist et al., 1977; Oldfield et al., 1978; Seelig & Seelig, 1978). An alternative method is the biosynthetic incorporation of deuterated fatty acids or of other deuterated substrates into a biological membrane as has been demonstrated for membranes of *Acholeplasma laidlawii* (Oldfield et al., 1972; Stockton et al., 1977; Smith et al., 1979) and *Escherichia coli* (Davis et al., 1979; Gally et al., 1979; Kang et al., 1979; Nichol et al., 1979). The latter approach has the advantage that the spectroscopic results are derived from intact biological membranes and that little biochemical membrane manipulation is involved. On the other hand, the lipid composition of biological membranes is generally rather complex and biosynthetic incorporation of deuterated fatty acids will label all lipid classes indiscriminately. This complicates the quantitative comparison between the native membranes and pure lipid model systems of well-defined chemical composition.

In the present study we have tried to minimize the problem of lipid heterogeneity by employing an *E. coli* double mutant (strain T2 GP) which is a fatty acid auxotroph and is also deficient in the synthesis of cardiolipin (Pluschke et al., 1978). *E. coli* membranes of strain T2 GP contain only two types of phospholipids, namely, phosphatidylethanolamine (~80 mol %) and phosphatidylglycerol (~20 mol %). Furthermore, if grown on elaidic acid (*trans*-Δ^9-octadecenoic acid), 80–95% of the natural fatty acids are replaced by this synthetic analogue without impairing the viability of the cells. By contrast, externally supplied palmitic acid or oleic acid (*cis*-Δ^9-octadecenoic acid) is incorporated to a much lesser extent [~50 mol %; cf. Gally et al. (1979)]. Most experiments reported here have therefore been performed with elaidic-*9,10-d*$_2$ acid which, for the purpose of ^{2}H NMR,[1] has the additional advantage that the two deuterons of the trans double bond give rise to identical quadrupole splittings due to symmetry reasons (Seelig & Waespe-Šarčevič, 1978). Inner and outer membranes of elaidate-enriched cells have been separated, and the structural properties of the intact membranes can thus be compared to those of model membranes composed of either extracted membrane phospholipids or synthetic phospholipids. Special attention is given to the existence of nonbilayer phases in *E. coli* membranes since this problem has been raised in two recent studies (Cullis & de Kruijff, 1978a; Davis et al., 1979).

Materials and Methods

Most of the experimental conditions have already been described in the previous paper and are not repeated here (Gally et al., 1979). Selectively deuterated elaidic acid and oleic acid were prepared as described previously (Seelig & Waespe-Šarčevič, 1978).

1,2-Bis(elaidoyl-*9',10'-d*$_2$)-*sn*-glycero-3-phosphoethanolamine was synthesized by the base exchange reaction of phospholipase D from the corresponding 1,2-bis(elaidoyl-

† From the Department of Biophysical Chemistry, Biocenter, University of Basel, CH-4056 Basel, Switzerland (H.U.G. and J.S.), and the Max-Planck-Institut für Biologie, Tübingen, West Germany (P.O. and G.P.). *Received October 5, 1979.* This work was supported by Swiss National Science Foundation Grant No. 3.409.78.

[1] Abbreviations used: NMR, nuclear magnetic resonance; Pipes, 1,4-piperazinediethanesulfonic acid; Brij 35, poly(oxyethylene) dodecyl ether; DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine.

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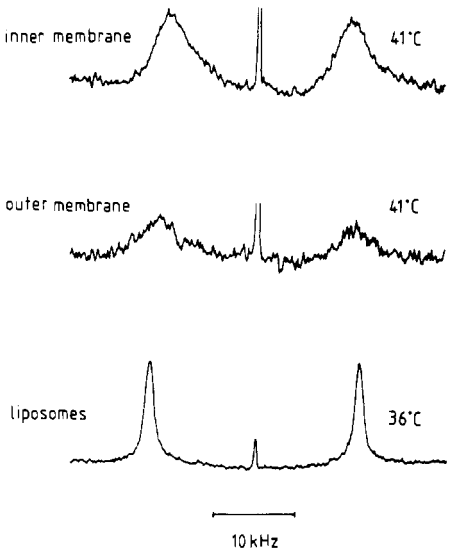

FIGURE 1: ^2H NMR spectra at 61.4 MHz of inner and outer membranes of *E. coli* T2 GP strain and derived liposomes enriched in elaidic-*9,10-d*$_2$ acid. Inner membrane at 41 °C: 0.01 M $NaPO_4$, pH 7.0, 0.1 M NaCl, and 0.01 M $MgCl_2$. Measuring time ~80 min. Outer membrane at 41 °C: same buffer as above. Measuring time ~40 min. Liposomes formed from the extracted phospholipids at 36 °C: 0.01 M $NaPO_4$, pH 7.0, 0.1 M NaCl, 0.001 M EDTA, and no $MgCl_2$. Measuring time 5 min.

9',10'-d$_2$)-*sn*-glycero-3-phosphocholine (Seelig & Gally, 1976). The latter compound was prepared by the method of Gupta et al. (1977).

Inner and outer membranes of elaidate-enriched membranes were separated according to established procedures (Osborn et al., 1972; Overath et al., 1975). The total lipids were extracted from the cells according to Ames (1968), and the pure phospholipids were recovered from a silicic acid column. Phosphatidylethanolamine and phosphatidylglycerol were separated on a DEAE-cellulose column (Rouser et al., 1969). The phospholipids were dispersed at 50 °C in a 0.01 M $NaPO_4$ or Pipes buffer solution of pH 7.0, containing 0.1 M NaCl and 0.001 M EDTA. The samples were equilibrated with the same buffer solution containing no EDTA but 0.002 or 0.01 M $MgCl_2$ in order to study the effect of Mg^{2+} ions. The Mg^{2+}/phospholipid molar ratio was always larger than unity. Phase transition temperatures and enthalpies were measured on a Mettler TA 2000 system. ^{31}P NMR measurements were made at 36.4 MHz under proton decoupling conditions as described earlier (Gally et al., 1975; Niederberger & Seelig, 1976). ^2H NMR measurements were made at 13.8 and 61.4 MHz as detailed elsewhere (Gally et al., 1979).

Results

Inner and Outer Membranes of E. coli T2 GP Cells. Figure 1 represents ^2H NMR spectra at 61.4 MHz of the inner and outer membranes of *E. coli* with the natural fatty acids being replaced to more than 85 mol % by elaidic-*9,10-d*$_2$ acid. The spectra of cells (not shown) are practically indistinguishable from those of the inner membrane. The ^2H NMR spectrum of the corresponding bilayer of *E. coli* lipids extracted from whole cells is also shown in Figure 1. In all three systems only one pair of lines is observed and the spectra are characteristic of phospholipids moving in a highly fluid but partially ordered environment (liquid-crystalline phase). From the separation of the lines, $\Delta\nu_Q$, the deuterium order parameter, $|S_{CD}|$, can be derived as described in the preceding paper (Gally et al., 1979). In this paper we will analyze the ^2H NMR spectra of the various systems in terms of this $|S_{CD}|$ parameter. Only

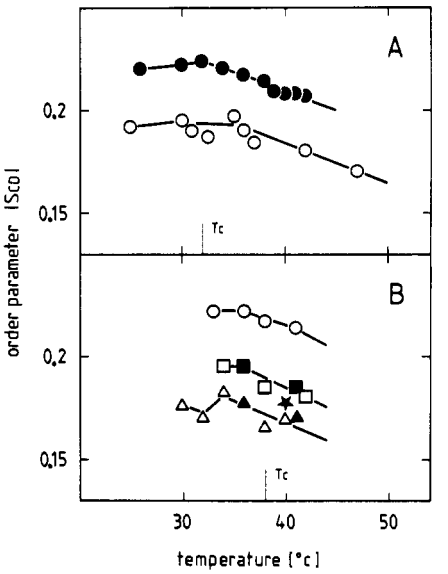

FIGURE 2: Temperature dependence of the deuterium order parameter, $|S_{CD}|$, of elaidate-*9,10-d*$_2$-enriched *E. coli* membranes and derived liposomes. (A) Influence of Mg^{2+} ions on *E. coli* lipid extract (~80 mol % phosphatidylethanolamine; ~20 mol % phosphatidylglycerol; ~93 mol % elaidic acid). (○) Liposomes in the absence of Mg^{2+} ions (buffer: 0.1 M NaCl, 0.01 M $NaPO_4$, and 0.001 M EDTA, pH 7.0). (●) Liposomes: no EDTA, instead 0.01 M $MgCl_2$. The gel to liquid-crystal phase transition of the Mg^{2+}-free liposomes is centered around 32 °C as determined by differential thermal analysis. (B) Comparison between *E. coli* T2 GP cells, inner and outer membranes, and derived liposomes. (○) Liposomes in 0.1 M NaCl, 0.01 M $NaPO_4$, and 0.001 M EDTA, pH 7.0 (79 mol % phosphatidylethanolamine; 21 mol % phosphatidylglycerol; ~86 mol % elaidic acid). The phase transition is centered around 38 °C as revealed by differential thermal analysis. The upward shift by 6 °C compared to (A) can be explained by a reduced elaidic acid content and by a slightly different ratio of phosphatidylethanolamine to phosphatidylglycerol. Outer membrane (□) and inner membrane (△) in Mg^{2+}-free buffer. Outer membrane (■) and inner membrane (▲) in the presence of 0.01 M $MgCl_2$ and no EDTA. Whole cells (*) in the presence of 0.01 M $MgCl_2$.

lipids in the fluid state are detected in the present experiments while gel-state lipids are not observed. The broad gel to liquid-crystal phase transition of *E. coli* membranes, which has been established by a variety of physical techniques (Overath & Träuble, 1973; Overath et al., 1975; Overath & Thilo, 1978), is thus reflected only in a change of the signal-to-noise ratio of the ^2H NMR spectra. At high temperatures (~40 °C) ^2H NMR spectra are relatively easy to record since most if not all lipids are in the fluid state [cf. Overath et al. (1975), Davis et al. (1979), and C. P. Nichol, J. H. Davis, G. Weeks, and M. Bloom, unpublished experiments]. At low temperatures (~30 °C) the major part of the lipids is in the gel state and the signal-to-noise ratio decreases considerably.

The deuterium order parameters, $|S_{CD}|$, of cells, inner and outer membranes, and extracted lipids are compared in Figure 2 and are found to increase in the order inner membrane < outer membrane < pure phospholipid bilayer. The differences between the inner and outer membranes are rather small. Neglecting these differences, the deuterium order parameters of both membranes can be approximated by those of whole cells, avoiding the rather cumbersome separation of inner and outer membranes. We have taken advantage of this fact in the preceding paper where all results were obtained with whole *E. coli* cells (Gally et al., 1979). The most interesting result of Figure 2 is the finding that the hydrocarbon chains are better ordered in a bilayer without protein than with protein. This difference is further augmented in the presence of 0.01 M $MgCl_2$. Surprisingly, the $|S_{CD}|$ parameters of inner and

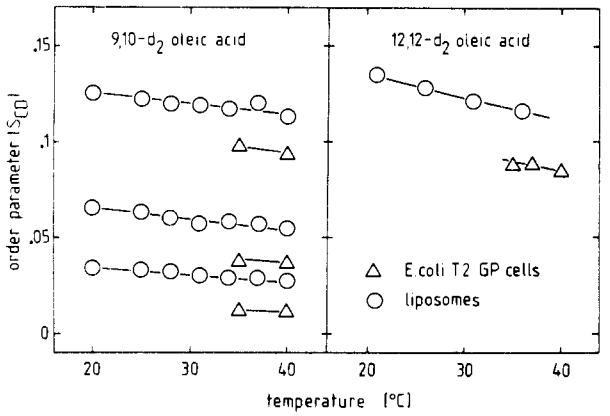

FIGURE 3: Deuterium order parameter, $|S_{CD}|$, of *E. coli* T2 GP strain grown in the presence of oleic-*9,10-*d_2 acid and oleic-*12,12-*d_2 acid. Spectra of oleate-*9,10-*d_2-enriched membranes and derived liposomes have been shown in the preceding paper (Gally et al., 1979): 0.01 M $NaPO_4$, pH 7.0, 0.1 M NaCl, and 0.01 M $MgCl_2$. (○) Liposomes; (△) whole cells.

outer membranes or whole cells are *not* changed by the addition of Mg^{2+} ions, whereas $|S_{CD}|$ increases by some 10–20% in the extracted phosphatidylethanolamine–phosphatidylglycerol mixture (Figure 2). Similar results have been obtained for *E. coli* T2 GP cells grown in the presence of oleic-*9,10-*d_2 acid and oleic-*12,12-*d_2 acid. The data are summarized in Figure 3. For the reasons given above, inner and outer membranes have not been separated in the latter experiments. Again, the $|S_{CD}|$ parameter is smaller in intact cells than in the phospholipid extract. The disordering influence of membrane proteins is therefore not limited to elaidate-enriched membranes but is also found in membranes which closely mimic wild type membranes [cf. Overath & Thilo (1978)].

Bilayer Membranes of Synthetic and Natural Phosphatidylethanolamine. The elaidate-enriched lipids of the strain studied here have 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE) as their major chemical species (~60–70 mol % of the total lipid). We have therefore synthesized DEPE selectively deuterated at the 9 and 10 positions of both fatty acyl chains. When dispersed in excess buffer (0.1 M NaCl and 0.01 M $NaPO_4$, pH 7.0), DEPE forms liquid-crystalline bilayers at temperatures $T_c \geq 35$ °C (Jackson & Sturtevant, 1977). Above T_c ^2H NMR spectra are readily obtained whereas below T_c the ^2H NMR signal is rather broad and distorted due to excessive line broadening.

In addition to the relatively abrupt order–disorder transition at $T_c = 35$ °C, DEPE undergoes a more gradual transformation from a lamellar to a hexagonal liquid-crystalline phase at temperatures between 45 and 60 °C. This transformation is quite obvious from the ^2H and ^{31}P NMR spectra shown in Figure 4 and has been investigated independently of us by Cullis & de Kruijff (1978a). At low temperatures (35–45 °C) the ^{31}P spectra are characterized by a large negative shielding anisotropy and are typical for a lipid bilayer (Gally et al., 1975). With increasing temperature this signal gradually disappears. It is replaced by a spectrum in which the chemical shielding anisotropy has a positive sign and is reduced in size by a factor of 2. This is exactly the spectral transformation one would predict for a lamellar → hexagonal phase transition if the molecular details of the head group motion are not affected by this transition (Seelig, 1978; Cullis & de Kruijff, 1978b). The coexistence of a lamellar and a hexagonal phase is revealed even better by the ^2H NMR spectra of Figure 4. At low temperatures only one pair of lines is observed and the quadrupole splitting ($\Delta\nu_Q \approx 25$ kHz) is comparable to that

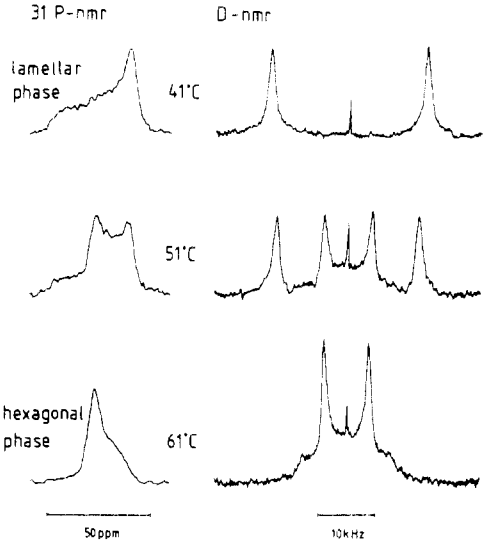

FIGURE 4: Lamellar → hexagonal liquid-crystal phase transition for synthetic DEPE (1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine) deuterated at the 9 and 10 positions of both elaidic acyl chains. DEPE is dispersed in excess buffer (0.01 M $NaPO_4$, pH 7.0, 0.1 M NaCl, and 0.001 M EDTA). The figure shows ^{31}P NMR (at 36.4 MHz) and ^2H NMR (at 61.4 MHz) spectra: 41 °C, lamellar phase; 51 °C, coexistence of lamellar and inverted hexagonal phases; 61 °C, inverted hexagonal phase.

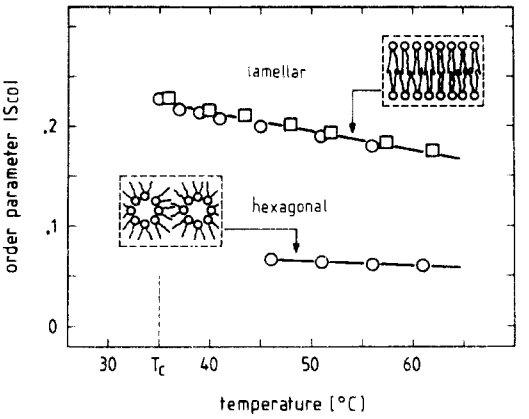

FIGURE 5: Comparison of natural phosphatidylethanolamine (□) extracted and purified from elaidate-*9,10-*d_2-enriched *E. coli* cells and synthetic 1,2-bis(elaidoyl-*9′,10′-*d_2)-*sn*-glycero-3-phosphoethanolamine (○). Variation of the deuterium order parameter, $|S_{CD}|$, with temperature. The synthetic phosphatidylethanolamine exhibits a lamellar → hexagonal phase transition between 40 and 60 °C, and the $|S_{CD}|$ parameter of both mesophases is given in the figure. According to differential thermal analysis, this phase transition is shifted to 80–90 °C in the natural phosphatidylethanolamine and was not investigated by ^2H NMR. In addition, both lipids show a gel to liquid-crystal transition centered around 35–37 °C.

found in related bilayer systems such as, for example, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine (Seelig & Waespe-Šarčevič, 1978). In the intermediate temperature range (45–60 °C) the spectrum consists of two quadrupole splittings and the intensity of the smaller quadrupole splitting of the hexagonal phase grows at the expense of the lamellar signal. Above 60 °C only the small quadrupole splitting associated with the hexagonal phase is seen. The variation of the deuterium order parameter, $|S_{CD}|$, with temperature for the lamellar and the hexagonal phase is summarized in Figure 5.

We may now compare synthetic DEPE with natural phosphatidylethanolamine, isolated from elaidate-*9,10-*d_2-enriched *E. coli* membranes. The predominant fatty acid in the natural lipid is, of course, elaidic acid (~90 mol %), but the

lipid also contains some lauric acid (~1 mol %), myristic acid (~5 mol %), and palmitic acid (~3 mol %). This small change in the fatty acid composition is sufficient to stabilize the bilayer structure. In contrast to synthetic DEPE, the natural phosphatidylethanolamine exhibits no lamellar → hexagonal phase transition in the temperature range investigated (30–75 °C). In other respects the physical behavior of the natural phosphatidylethanolamine is similar to that of its synthetic analogue. The gel to liquid-crystal phase transition occurs at ~37 °C as determined by differential thermal analysis but is distinctly broadened compared to DEPE. The deuterium order parameters are found to be almost identical for the two lipids as shown in Figure 5. Both, synthetic DEPE and natural phosphatidylethanolamine, have also been dispersed in buffer containing 0.01 M $MgCl_2$ (buffer composition 0.1 M NaCl, 0.01 M $MgCl_2$, and 0.01 M $NaPO_4$, pH 7.0). The hydrocarbon chain ordering as well as the temperature-dependent phase behavior of both systems is not affected in any obvious manner by the addition of 10 mM $MgCl_2$.

Mixtures of Phosphatidylethanolamine and Phosphatidylglycerol. The natural phospholipid composition of the *E. coli* T2 GP mutant is about 80 mol % phosphatidylethanolamine and 20 mol % phosphatidylglycerol. We have therefore investigated the structural properties of mixtures of the two lipids, again extracted and purified from elaidate-enriched *E. coli* T2 GP mutant. The fatty acid composition was 93 mol % elaidic-*9,10-*d_2 acid, the rest being saturated fatty acids of shorter chain length. Differential thermal analysis revealed a relatively broad gel to liquid-crystal phase transition between 26 and 36 °C with a midpoint transition temperature of T_c = 32 °C (multilamellar liposomes in 0.1 M NaCl and 0.01 M $NaPO_4$, pH 7.0). A second transition with a much smaller enthalpy change was observed at ~80 °C. This high-temperature transition probably represents a lamellar → hexagonal phase transition, but no X-ray studies are available as yet.

The interesting new feature of the mixture compared to natural or pure synthetic phosphatidylethanolamine is its sensitivity to Mg^{2+} ions. Addition of Mg^{2+} ions at concentrations of 10 or even 2 mM leads to an *increase* in the quadrupole splitting of the mixture without any detectable change in the apparent line width. This effect is quantitated in Figure 2 (upper panel) which shows the temperature dependence of the deuterium order parameter $|S_{CD}|$ with and without Mg^{2+}. The Mg^{2+} effect is reversible, and removal of Mg^{2+} by washing the multilamellar dispersion with 10 mM EDTA solution restores the spectrum of the Mg^{2+}-free bilayer.

Discussion

Inner and outer membranes of *E. coli* strain T2 GP exhibit ^2H NMR spectra which are qualitatively rather similar. They are characterized by relatively broad deuterium resonances while the apparent line width of the pure phospholipid membrane is much narrower. The broad lines could be an expression of the membrane heterogeneity. More than 200 different proteins have been identified in *E. coli* membranes, and it is reasonable to assume that the microenvironment varies slightly from protein to protein. Since the membranes are highly fluid, the phospholipid molecules could jump rather rapidly between the various domains. The jump rate is fast enough to average the differences in the quadrupole splittings but is still too slow to ensure a complete line narrowing in this multisite exchange (cf. Davis et al. (1979)].

Quantitatively, the deuterium order parameter of elaidate-*9,10-*d_2-enriched outer membranes ($|S_{CD}|$ = 0.19, 38 °C) is increased compared to inner membranes at the same temperature ($|S_{CD}|$ = 0.17). This is in agreement with a related study of Davis et al. (1979) where *E. coli* (strain L 51) was grown in the presence of perdeuterated palmitic acid. From studies with selectively deuterated phospholipids it is known that the deuterium order parameter varies considerably with the chain position; it is approximately constant for the first 8–10 carbon atoms and decreases rapidly as the site of deuteration approaches the terminal methyl group (Seelig & Seelig, 1974; Seelig & Browning, 1978). This characteristic signature of phospholipid membranes is also reflected in the ^2H NMR spectra of perdeuterated *E. coli* membranes which contain a larger number of inequivalent deuterium sites. For perdeuterated palmitate-enriched outer membranes Davis et al. (1979) estimated $|S_{CD}|_{plateau} \approx 0.26$ (37 °C) and for the inner membranes $|S_{CD}|_{plateau} \approx 0.20$ (34 °C). Again the outer membrane is found to be better ordered than the cytoplasmic membrane. Davis et al. (1979) ascribed the differences in the order profiles to different phase transition temperatures of the two membranes. The phospholipid components of *E. coli* membranes undergo a relatively broad gel to liquid-crystal phase transition [Overath & Träuble, 1973; Overath et al., 1975; for a review, see Overath & Thilo (1978)], and the ^2H NMR spectra of perdeuterated palmitate-enriched membranes suggest that the phase transition of the inner membrane occurs over a region ~7 °C lower in temperature than that of the outer membrane. This result has also been obtained by incorporation of selectively deuterated palmitic acid (Nichol et al., 1979). A similar explanation may hold true for the elaidate-enriched membranes investigated in this study. If the $|S_{CD}|$ vs. temperature data for the inner membrane (Figure 2) are displaced by +8 °C, they overlap those of the outer membrane. However, since such a difference was not observed by X-ray diffraction and fluorescence techniques (Overath et al., 1975), it may equally be possible that the phase transitions of elaidate-enriched inner and outer membranes occur at the same temperature and that the differences in the ordering profiles are caused by some other structural properties of as yet unknown origin.

If compared to pure phospholipid bilayers formed from *E. coli* lipids, the fatty acyl chains of inner and outer membranes of elaidate or oleate-enriched *E. coli* cells are found to be less ordered by ~10–20%. A similar result has been reported by Kang et al. (1979), who incorporated palmitic-ω,ω,ω-d_3 acid into *E. coli* fatty acid auxotrophs and observed a reduction of the quadrupole splitting from 3.4 kHz for the extracted lipids to 2.2 kHz for intact cells. In addition, a reduction of the deuterium quadrupole splitting has been noted for a variety of reconstituted lipid–protein membranes (Oldfield et al., 1978; Seelig & Seelig, 1978).

The results obtained for *E. coli* membranes can be interpreted in close analogy with the model suggested for the interaction of cytochrome *c* oxidase with a phospholipid bilayer (Seelig & Seelig, 1978). In short, the protein molecules are assumed to have a regular rugged surface due to the protruding amino acid side groups. In the fluid membrane the flexible fatty acyl chains will fill the space between the side groups in order to create a densely packed lipid–protein bilayer. This entails two consequences. First, the hydrocarbon chains become distorted, the amplitudes of the angular excursions of the methylene segments increase, and the $|S_{CD}|$ parameter is reduced. Second, as a consequence of the more distorted chain conformation, the friction between the segments must increase and the membrane fluidity will decrease. The incorporation of proteins into a lipid bilayer would thus produce more disordered fatty acyl chains and, at the same time, a more viscous bilayer. The postulated viscosity increase must still be dem-

onstrated experimentally for *E. coli* membranes, but measurements on other lipid–protein systems support this contention [e.g., Cherry et al. (1977) and Stoffel et al. (1977)]. At present, it suffices to differentiate clearly between the structural and the dynamic aspects of lipid–protein interaction, since these are independent physical parameters. No conclusions can be reached from the $|S_{CD}|$ parameter concerning the bilayer fluidity or chain mobility. $|S_{CD}|$ is related to the average chain conformation but not to the rate of conformational changes.

The protein-induced disorder appears to be rather small if compared, for example, to the disorder induced by the gel to liquid-crystal phase transition where $|S_{CD}|$ changes from 0.5 in the gel state to ~0.2 in the fluid state. This suggests that the forces determining the nonspecific lipid–protein interactions in the fluid membrane are quite similar to those characteristic of lipid–lipid interactions and are mainly steric repulsion and van der Waals attraction forces.

The occurrence of a lamellar → hexagonal phase transition in pure DEPE model membranes suggests that alternative structures other than the lamellar phase could regulate specific membrane functions. Theoretically, the change in the phase geometry from lamellar to hexagonal should be accompanied by a reduction of the quadrupole splitting and of the deuterium order parameter by exactly a factor of 2 if the average conformation of the hydrocarbon chains remains unaltered (Seelig, 1977). The experimentally observed reduction is, however, larger and amounts to almost a factor of 3. The hydrocarbon interior of the hexagonal phase thus appears to be distinctly less ordered than that of the lamellar phase, at least at the level of the trans double bond. At the level of the phosphate group no changes are observed. This difference can be explained by the different packing constraints imposed by the geometry of the two phases. X-ray investigations suggest that unsaturated phosphatidylethanolamines have a strong tendency to form *inverted* hexagonal phases (Rand et al., 1971). In the inverted hexagonal phase a cylindrical core is made up from water molecules and the inner aqueous cylinder is surrounded by the lipid polar groups with the fatty acyl chains facing outward, forming a semiliquid hydrocarbon environment between the aqueous rods (cf. Figure 5). The anchoring of the polar groups in the lipid–water interface can be expected to be similar or identical in both the lamellar and the inverted hexagonal phase, but the hydrocarbon chains of the hexagonal phase must be packed less regularly than those of the bilayer phase. This gain in configurational freedom is reflected in the reduction of the deuterium order parameter beyond the geometric factor 2. Our findings for DEPE appear to be in contrast to those obtained for simple soaplike systems (Mely et al., 1975) and provide the first definite evidence that the chain conformation is different in lamellar and nonlamellar phases.

It has been speculated recently that nonbilayer phases may constitute important regulatory elements of biological membranes (Stier et al., 1978; de Kruijff et al., 1978). Cullis & de Kruijff (1978a) have obtained evidence from ^{31}P NMR that *E. coli* phosphatidylethanolamine exhibits a reversible bilayer → hexagonal phase transition at 55–60 °C. Davis et al. (1979) also observed a nonbilayer component in both *E. coli* cell membranes and derived liposomes which has the characteristics of a hexagonal phase. In the latter experiments the contribution of the hexagonal component to the total spectral intensity increases with time, suggesting to the authors that the hexagonal phase is associated with some degradation of the *E. coli* cells. In the present experiments the ^2H NMR spectra of whole *E. coli* cells or extracted *E. coli* phosphatidylethanolamine do not provide evidence for the formation of nonbilayer phases. This may be related, in part, to the *E. coli* strain employed in this study, but the result is not at all surprising since most of the *E coli* phospholipids must indeed be organized in a conventional bilayer in order to maintain the membrane-supporting matrix. Likewise, in a more recent study Nichol et al. (1979) also found no evidence for nonlamellar phases in *E. coli*.

Of biological interest is the observation that Mg^{2+} ions influence the phospholipid organization of the extracted *E. coli* lipids but not that of the native membrane. Calorimetric experiments have shown a marked effect of Mg^{2+} ions on the thermotropic behavior of synthetic phosphatidylglycerol bilayers, leading to a crystallike structure at ion concentrations larger than 1 $Mg^{2+}/2$ phospholipid molecules (Ververgaert et al., 1975; Van Dijck et al., 1975). On the other hand, the gel to liquid-crystal phase transition of a Mg^{2+}-free phosphatidylglycerol bilayer occurs some 20 °C lower than that of phosphatidylethanolamine with the same fatty acid composition [cf. Van Dijck et al. (1976) and Findlay & Barton (1978)]. This, together with the above observation that Mg^{2+} has no influence on pure phosphatidylethanolamine bilayers, suggests the following explanation for the results shown in Figure 2. Addition of phosphatidylglycerol to a phosphatidylethanolamine bilayer of similar fatty acid composition will lower the gel to liquid-crystal transition temperature and will induce a more disordered hydrocarbon interior. This effect is reversed by the addition of Mg^{2+} ions which remove phosphatidylglycerol from the lipid mixture by formation of quasi-crystalline Mg^{2+}–phosphatidylglycerol clusters. The structure of the remaining phosphatidylethanolamine bilayer is not affected by these clusters, and the hydrocarbon chain ordering is the same as that of a pure phosphatidylethanolamine bilayer.

This interpretation is strengthened by two experiments with phosphatidylglycerol–phosphatidylethanolamine model systems in which only one class of lipid molecules has been deuterated with elaidic-*9,10-*d_2 acid. In the first experiment, deuterated phosphatidylethanolamine (50 mol %) is mixed with nondeuterated phosphatidylglycerol (50 mol %), both purified from elaidate-enriched *E. coli* T2 GP phospholipids. With and without Mg^{2+}, the ^2H NMR spectra exhibit the characteristic bilayer pattern. The quadrupole splitting is increased by the addition of Mg^{2+}, in agreement with Figure 2. A different result is, however, obtained in the reverse experiment using nondeuterated phosphatidylethanolamine (79 mol %) and deuterated phosphatidylglycerol (21 mol %). Here ^2H NMR signal can only be observed for the Mg^{2+}-free bilayer dispersion. Addition of 10 mM $MgCl_2$ completely eliminates the deuterium spectrum, indicating the formation of a crystalline Mg^{2+}–phosphatidylglycerol complex. After the mixture was washed with EDTA solution, the original deuterium quadrupole splitting reappears.

From the above model studies with the phosphatidylglycerol–phosphatidylethanolamine mixture, it is clear that the Mg^{2+} effect is mediated via phosphatidylglycerol. Therefore, the invariance of the ^2H NMR spectra of *E. coli* membranes against changes in the Mg^{2+} concentration indicates that phosphatidylglycerol is not accessible to Mg^{2+} in the native *E. coli* membrane. A similar effect has also been reported for the interaction of spectrin–actin with 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (Mombers et al., 1977). Among the various possibilities of explanation, it is most tempting to speculate that phosphatidylglycerol is

shielded from the action of Mg^{2+} by being bound to some membrane proteins. In order to elucidate in more detail the exact mode of this protective interaction, we are at present selectively deuterating the glycerol polar group in *E. coli* cells.

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