Study of the Cytoplasmic and Outer Membranes of *Escherichia coli* by Deuterium Magnetic Resonance†

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ABSTRACT: The cytoplasmic and outer membranes of *Escherichia coli* were studied between 0 and 40 °C by deuterium magnetic resonance quadrupolar echo spectroscopy. The L51 strain of *E. coli* was used to incorporate perdeuterated palmitic acid into the membrane phospholipids. The cytoplasmic and outer membranes were separated using standard techniques. The spectrum of each membrane preparation was dominated at high temperatures (≥37 °C) by the characteristic liquid-crystalline plateau previously observed for perdeuterated palmitate chains in model phospholipid membranes. At low temperatures, the shape and width of the spectrum were characteristic of the gel phase. The relative intensities of the liquid-crystalline and gel features varied systematically with temperature. A quantitative analysis of the acyl chain orientational order was carried out by using the method of moments. The orientational order at each temperature was greater in the outer membrane sample than in that of the cytoplasmic membrane, indicating that the liquid-crystalline–gel transition region in the outer membrane is shifted to higher temperatures than that of the cytoplasmic membrane by about 7 °C. It is clear from the results that most of the phospholipid molecules participate in the phase transition.

Escherichia coli possess two distinct membranes with different compositions and physiological properties. While the fatty acid compositions of the cytoplasmic (or inner) and outer membrane lipids are similar (Lugtenberg & Peters, 1976; Overath et al., 1975), their different lipid to protein ratios and the presence of lipopolysaccharide in the outer membrane suggest that there might be a significant difference in the fluidity characteristics of the two membranes.

It is now well established that the phospholipid components in the cytoplasmic membrane of *E. coli* undergo a relatively broad gel to liquid-crystalline phase transition. This phase transition has been detected by a variety of different techniques including differential scanning calorimetry (Baldassare et al., 1976; Jackson & Sturtevant, 1977), X-ray diffraction (Overath et al., 1975; Schechter et al., 1974; Linden et al., 1977), electron spin resonance (ESR)[1] (Linden et al., 1973; Sackmann et al., 1973), and fluorescence (Overath & Träuble, 1973; Cheng et al., 1974; Overath et al., 1975). The use of fatty acid auxotrophs has demonstrated that there is a correlation between the temperature at which changes in physiological functions (such as transport processes) occur and the phase transition temperature[2] of the membrane phospholipids (Linden et al., 1973; Schechter et al., 1974; Cronan & Gelmann, 1975). Finally, the X-ray and fluorescence studies have led to the interpretation that, relative to the corresponding extracted lipids, only ~60–80% of the phospholipid hydrocarbon chains participate in the phase transition (Schechter et al., 1974; Träuble & Overath, 1973; Overath et al., 1975), suggesting that the remaining phospholipid interacts with membrane protein.

The outer membrane of *E. coli* has been less extensively studied by physical methods. The outer membrane has a larger percentage of saturated fatty acids and phosphatidylethanolamine (Lugtenberg & Peters, 1976), and the phospholipid to protein ratio for the outer membrane is less than half that of the cytoplasmic membrane. The interpretation of the X-ray and fluorescence studies by Overath et al. (1975) was that the two membranes had phase transitions occurring over the same temperature range. In their interpretation, the main differences between the cytoplasmic and outer membrane samples was that only 25–40% of the phospholipid of the outer membrane participated in the phase transition (compared with 60–80% for the cytoplasmic membrane) (Overath et al., 1975), suggesting that the remaining phospholipid interacts with either membrane-bound protein or lipopolysaccharide. The ESR study of Rottem & Leive (1977) indicated that the hydrocarbon chain mobility was more restricted in the outer than in the cytoplasmic membrane. A recent ESR study of the cytoplasmic and outer membranes of another Gram-negative bacterium, *Salmonella typhimurium* (Nikaido et al., 1977), indicated that the phase transitions of the two membranes were nearly identical. While there appears to be general agreement that both the cytoplasmic and outer membranes of *E. coli* undergo a phase transition over a similar temperature range, there is little agreement with regard to the detailed variation with temperature of the hydrocarbon chain mobility in the two membranes.

The use of these physical techniques in the study of *E. coli* membranes has provided a useful picture of the membrane phase transition. Deuterium nuclear magnetic resonance (^{2}H NMR), which has provided an accurate and detailed description of model membranes (Seelig & Seelig, 1977; Davis, 1979), can now be applied to the study of deuterium-labeled, natural membrane systems (Stockton et al., 1977). The increase in the entropy of a bilayer membrane passing from the gel to liquid-crystalline phase is almost completely associated with an increase in orientational disorder of the acyl chains above the phase transition (Phillips, 1972). The deuterium

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[1] Abbreviations used: ESR, electron spin resonance; ^{2}H NMR, deuterium magnetic resonance; PPBE, proteose peptone–beef extract, contained 1% proteose peptone no. 3 (Difco), 0.1% w/v beef extract, 0.5% w/v NaCl; KDO, 2-keto-3-deoxyoctonate; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance, DEGS, diethylene glycol succinate.

[2] Strictly speaking, it is not possible to define a "transition temperature" for the gel to liquid-crystalline phase transition in biological membranes where gel and liquid-crystalline regions of the sample coexist over a wide temperature range.

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magnetic resonance spectrum gives a direct measure of this orientational order in terms of the deuterium quadrupolar splitting. The variation of acyl chain order with chain position, as determined by ^2H NMR, provides a characteristic signature of the fluid membrane. The positions on the top half of the chain, that part which is closer to the polar head group, show approximately the same degree of order, forming the "plateau", while a rapid decrease in order is observed as the position of deuteration is moved closer to the terminal methyl group. This variation is characteristic of both biological (Stockton et al., 1977) and model membranes (Seelig & Seelig, 1977). The ^2H NMR spectrum obtained below the phase transition for both model and biological systems is characterized by much larger quadrupolar splittings than are found in the liquid-crystalline phase.

The quadrupolar echo technqiue (Davis et al., 1976) allows one to obtain essentially distortion free spectra for biological samples where the signals are weak and the spectra are broad and often complex. The use of this technique along with a recently developed moment method of analyzing complex deuterium spectra (Bloom et al., 1978) has made it possible to obtain a quantitative measure of fluidity in systems in which the acyl chains of the phospholipid molecules are completely deuterated and to study the variation in chain order in both the gel and liquid-crystalline phase as well as throughout the phase transition region (Davis, 1979). Using these two new techniques, we have attempted to establish quantitative relationships between the fluidity of the cytoplasmic and outer membranes of *E. coli*, to correlate these findings with similar measurements on the lipids extracted from these membranes, and to compare the ^2H NMR spectra of the separated membrane preparations with those obtained from samples of intact cells.

Materials and Methods

Materials. Perdeuterated palmitic acid ($CD_3(CD_2)_{14}$-COOH) was prepared by the method of Hsiao et al. (1974) by using reagent grade palmitic acid purchased from Eastman Kodak. [U-^{14}C]Palmitic acid was obtained from New England Nuclear, Lachine, Quebec.

Strains and Growth Conditions. E. coli, strain L51 (formerly AB1623 fadE), incorporates exogenous fatty acid into membrane phospholipid and is defective in fatty acid oxidation (Silbert et al., 1973). The basic growth medium contained minimal salts (7 g/L K_2HPO_4, 3 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$) and 0.4% glycerol, 5 mM glutamate, 2 μg/mL thiamine, 0.5 μg/mL yeast extract, 0.1 mg/mL $MgSO_4 \cdot 7H_2O$, 0.5 μg/mL $FeSO_4 \cdot 7H_2O$, 1 mg/mL Brij 58, and 50 μg/mL perdeuterated palmitic acid. For spheroplast formation, it was found necessary to supplement this medium with 0.05% v/v proteose peptone–beef extract (PPBE) (Osborn & Munson, 1974).

Strain L51 grew exponentially in this medium for 2–3 generations to an absorbance at 600 nm of $\approx$0.3 and then grew exponentially at a reduced rate. During this second exponential phase, the cells clumped together and large amounts of cell debris were visible, suggesting that considerable cell lysis was occurring. In all experiments, cells were harvested before the end of the initial exponential growth.

The yield of cells in the initial growth phase was not increased by using a larger inoculum. In an attempt to increase yields and to produce membranes with an increased proportion of unsaturated fatty acid, the basic growth medium was supplemented for some experiments with PPBE, 4 g/L casein hydrolysate (Nutritional Biochemical Co., Cleveland) and oleic acid (50 μg/mL). Cells preadapted to this supplemented medium grew exponentially to an absorbance at 600 nm of $\approx$1.2.

Preparation of Intact Cells and Crude Envelope Fractions. Cells were grown in the basic growth medium and harvested at room temperature by centrifugation at 10000 rpm for 15 min by using a Sorvall GSA rotor. The cells were resuspended in minimal salts solution containing 1 mg/mL Brij 58 and recentrifuged. After a second wash with minimal salt solution, the cell pellet was used immediately for ^2H NMR studies.

A crude envelope fraction was obtained from washed cells by the method of Schnaitman (1970a,b). The pellet obtained by centrifugation at 47000 rpm for 45 min in a Beckman 60 Ti rotor was used for ^2H NMR and lipid analysis.

Preparation of Cytoplasmic and Outer Membranes. The method of Osborn & Munson (1974) was used. Spheroplasts were prepared at 0 °C from fresh cells harvested at room temperature and then lysed by a brief sonication at 0–5 °C. Any unlysed spheroplasts were removed by centrifugation and, after the membranes were collected and washed, they were separated by isopycnic centrifugation in a sucrose density gradient.

One membrane band was formed at ρ = 1.14–1.17 g/mL which corresponded to the cytoplasmic membrane and another was formed at ρ = 1.21–1.23 g/mL, corresponding to the outer membrane. These bands were carefully removed from the gradients, and a portion of each was retained for analysis of succinate dehydrogenase activity, 2-keto-3-deoxyoctonate (KDO) content, and lipid fatty acid composition. The remaining material was diluted with 1 mM EDTA to a sucrose concentration of approximately 8% and centrifuged at 47000 rpm in a Beckman 60 Ti rotor for 2 h. The membrane pellets were then transferred to NMR tubes. In some preparations, material was present at intermediate densities on the sucrose gradient. This material was probably a mixture of cytoplasmic and outer membranes and unseparated cell envelopes, as suggested by Osborn et al. (1972), and was discarded.

Lipid Analysis. Lipids were extracted from the membranes by a modification (Ames, 1968) of the method of Bligh & Dyer (1959). The lipid extracts were saponified as previously described (Gilkes & Weeks, 1977) and the saponifiable fraction was methylated with 10–15% BF_3 in methanol (Eastman Kodak) at 37 °C overnight. The methyl esters were extracted with pentane and separated by gas–liquid chromatography on a 12% DEGS column at 150 °C.

In some experiments, the lipid extract was fractionated into phospholipid, free fatty acid, and neutral lipid components by thin-layer chromatography (Brown & Johnston, 1962). The lipids were eluted from the plates as described by Skipski & Barclay (1969) and analyzed for fatty acid content as described above after the addition of arachidic acid as an internal standard.

Lipid Extraction. After the ^2H NMR spectra of the membranes were recorded, the samples were extracted by the modification of the method of Bligh & Dyer (1959). The $CHCl_3$ extract was dried down, redissolved in benzene, and dried overnight under vacuum. The samples were then resuspended in H_2O and thoroughly mixed.

Other Analytical Procedures. Protein content was measured by the method of Lowry et al. (1951). KDO content was determined as described by Osborn et al. (1972), and succinate dehydrogenase was assayed at 30 °C as described previously (Lee et al., 1975). Radioactivity in lipid extracts was determined in toluene scintillation fluid, while radioactivity in aqueous cell fractions was determined in Triton–toluene scintillation fluid.

NMR Techniques. The deuterium spectra were taken at 34.44 MHz in a high resolution superconducting solenoid supplied by Nalorac, Inc., Concord, CA, with a Bruker SXP 4-100 NMR spectrometer. The transient digitization and averaging were accomplished with a Nicolet 1090 AR digital oscilloscope interfaced to an Intel 8080A microprocessor-based data acquisition system. The Fourier transforms and moment analysis were done with a BNC-12 minicomputer.

All spectra were obtained by using the quadrupolar echo technique (Davis et al., 1976) with a pulse separation of 60 μs, with signal averaging for, typically, 3–4 h. No filters were used in the data acquisition, and no phase corrections were made to the transforms. When dealing with very broad spectra, such as those obtained in the gel phase of model or natural membranes, one finds it difficult to obtain a 90° pulse short enough to rotate all parts of the spectrum through the same angle. The resulting distortion of the spectrum can be severe. The second moments of the outer membrane spectra (obtained with 9-μs pulses), given by the open circles in Figure 6, were obtained by making a two-segment linear correction by using the values of the second moment of spectra obtained with 4-μs pulses at 0, 20, and 37 °C (closed circles in the figure). The 4–4.5-μs, 90° pulses used in most of these experiments introduce a small systematic error into the determination of the moments of the spectra. This error is negligible in the liquid-crystalline phase but is estimated to be $\lesssim$25% for the second moment at the lowest temperatures (Davis, 1979). It is possible to make a theoretical correction for this effect (M. Bloom, J. H. Davis, and M. I. Valic, unpublished results). Although we routinely calculate the first eight moments by integrating over the spectrum, the accuracy of the higher moments depends critically on the fidelity of the broad parts of the spectrum. For this reason, only the values of the first few moments of these spectra (M_1 to M_4) are reliable in the liquid-crystalline phase and at lower temperatures only M_1 and M_2 can be accurately determined.

The temperature of the sample was measured and controlled in a cylindrical copper oven enclosing the sample and radiofrequency coil. The temperature gradient across the sample volume is estimated to be less than 1 °C.

Description of Bilayer Order and Moment Analysis. In simple systems where only one type of deuterium site exists, such as in specifically deuterated acyl chains in model membrane systems, the ^{2}H NMR absorption signal for nonoriented samples exhibits a characteristic "powder" spectrum (Dahlquist et al., 1977, for example, or Abragam, 1961). The dominant features of this powder pattern are its two sharp peaks separated by an angular frequency $\omega_Q = (3e^2qQ/4\hbar)S_{CD}$, where $e^2qQ/\hbar$ is the quadrupolar coupling constant ($e^2qQ/\hbar = 2\pi \times 167$ kHz, for fatty acid methylene groups; Davis & Jeffrey, 1977), and $S_{CD} = 1/2\langle 3\cos^2\theta - 1\rangle$ is the C–D bond order parameter. θ is the angle between the C–D bond vector and the bilayer normal, and the angular brackets denote an average over the configurations of the acyl chain. The separation of the two peaks in the spectrum (the quadrupolar splitting) gives a direct measure of the C–D bond order parameter.

The most prominent feature of ^{2}H NMR spectra of *perdeuterated* acyl chains in the liquid-crystalline phase of phospholipid bilayer model membranes, such as dipalmitoyl-d_{31}-phosphatidylcholine (DPPC-d_{62}) (Davis, 1979), and of natural membranes, such as that of *Acholeplasma laidlawii* (see Figure 2a of Stockton et al., 1977), is the sharp edge associated with the peaks of the powder patterns of most of the methylene deuterons. The deuterons of the terminal methyl groups give a narrower readily identifiable contribution to the spectrum. The sharp edge of the spectra of these perdeuterated systems is a manifestation of the existence of a plateau in the variation of S_{CD} with chain position for both model (Seelig & Seelig, 1977) and biological membranes (Stockton et al., 1977; Seelig & Browning, 1978). Associated with each edge in these ^{2}H NMR powder pattern spectra is a less pronounced shoulder having a cut-off at twice the frequency difference between the edge and the center of the spectrum.

In deuterium-labeled phospholipids in biological membranes, there may be a large number of inequivalent deuterium sites. Most biological membranes have a variety of phospholipids with different polar head groups and different combinations of acyl chains of various lengths and degrees of unsaturation. These differences may give rise to variations in the value of S_{CD} among the different lipids even if all lipids were labeled at the same position. Also, at physiological temperatures biological membranes are often in a mixed phase containing regions of gel-phase lipid as well as liquid-crystalline lipid. There are large differences between the values of S_{CD} found in gel and liquid-crystalline regions of the sample. Finally, the interface between lipid and protein in a biological membrane may also give rise to observable variations in S_{CD}. The description of the orientational order of such a complex system requires not simply an order parameter S, but more generally, an order parameter distribution function $P(S)$ where, for a quasicontinuous distribution of S, $P(S)dS$ is the probability of finding an orientational order parameter between S and $S + dS$.

It is shown elsewhere (Bloom et al., 1978) that there is a straightforward method of characterizing $P(S)$ in terms of the moments of the ^{2}H NMR spectrum. Denoting the ^{2}H powder pattern line shape function by $f(\omega - \omega_0)$, where ω_0 is the nuclear Larmor angular frequency, the nth moment of the spectrum is given, with $x = \omega - \omega_0$, by eq 1. Here we have made use

$$M_n = \frac{\int_0^\infty x^n f(x)\,dx}{\int_0^\infty f(x)\,dx} \tag{1}$$

of the fact that $f(x) = f(-x)$ for first-order quadrupolar broadened ^{2}H NMR powder patterns. This allows us to make use of both the odd and even moments defined by eq 1 in terms of the half-spectrum corresponding either to $\omega \geq \omega_0$ or $\omega \leq \omega_0$.

Defining the corresponding moments of the order parameter distribution function $P(S)$ by eq 2, it is found that mea-

$$S_n = \int_0^\infty S^n P(S)\,dS \tag{2}$$

surement of M_n from $f(\omega)$ provides a unique way of determining S_n in terms of M_n (when broadening can be neglected) and an easily calculated constant A_n, i.e.

$$M_n = A_n \frac{3}{4}\left(\frac{e^2qQ}{\hbar}\right)^n S_n \tag{3}$$

In this paper we shall only be concerned with $n = 1$ and 2 for which $A_1 = 2/(3\sqrt{3})$ and $A_2 = 1/5$, giving $A_2/A_1^2 = 1.35$. Then we can define a parameter Δ_2 as

$$\Delta_2 = \frac{S_2 - S_1^2}{S_1^2} = \frac{M_2}{1.35M_1^2} - 1 \tag{4}$$

This parameter gives the relative mean square width of the

Table I: Characterization of Cytoplasmic and Outer Membrane Fraction of *E. coli* Strain L51

FA supplt[d]	succinate dehydrogenase[a] cyto-plasmic membr	succinate dehydrogenase[a] outer membr	KDO[b] cyto-plasmic membr	KDO[b] outer membr
palmitic acid[b]	0.59	0.04	0.02	0.14
palmitic acid + oleic acid[c]	0.55	0.04	0.01	0.08

[a] In μmol min^{-1} (mg of protein)$^{-1}$. [b] In μmol (mg of protein)$^{-1}$. [c] The basic growth medium was supplemented with PPBE. [d] The basic growth medium was supplemented with PPBE, casein hydrolysate, and oleic acid. [e] Abbreviations: FA, fatty acid; supplt, supplementation.

distribution of orientational order parameters. A system having only a single order parameter has the property that $S_n = S_1^n$, giving $\Delta_2 = 0$ (neglecting broadening). Larger values of Δ_2 imply wider ranges of S relative to the average order parameter S_1.

Results and Discussion

Incorporation of Palmitic Acid into Cellular Constituents. Cells were grown in the basic growth medium supplemented with PPBE and 0.01 μCi/mL of [U-^{14}C]palmitic acid. Over 97% of the fatty acid incorporated was in cellular lipid, suggesting that little, if any, degradation of the palmitic acid had occurred. The lipid extract was fractionated by thin-layer chromatography and it was found that 98.8% of the radioactivity was in phospholipid, while 1.2% was in free fatty acid. There was negligible radioactivity in the neutral lipid fraction.

In other experiments, cytoplasmic and outer membrane lipid extracts were prepared from cells grown in the basic medium supplemented with PPBE and were fractionated into phospholipid, free fatty acid, and neutral lipid. The amount of perdeuterated palmitic acid present in each fraction was determined by gas–liquid chromatography. It was found that 99% of the perdeuterated palmitic acid was present in the phospholipid fraction. These results demonstrated that essentially all of the deuterium in the cell was in the membrane phospholipids.

Silbert et al. (1973) have demonstrated that under similar conditions about 96% of the phosphatidylethanolamines (the predominant phospholipid) have a palmitoyl chain in the one position and only about 30% of these have a palmitoyl chain in the two position. For this reason the deuterated palmitic acid (assuming there has been no distinction between deuterated and protiated palmitic acid) uniformly represents essentially all of the phospholipid in the membrane.

Characterization of the Cytoplasmic and Outer Membrane Fractions. The succinate dehydrogenase specific activity of the cytoplasmic membrane fraction was approximately 14 times that of the outer membrane fraction. The KDO content of the outer membrane was seven to eight times that of the cytoplasmic membrane. These measurements, presented in Table I, indicate that the amount of cross-contamination was relatively slight (Hoekstra et al., 1976; Lugtenberg & Peters, 1976). A loss of lipopolysaccharide content of <10% occurred during preparation and sonication of the spheroplasts.

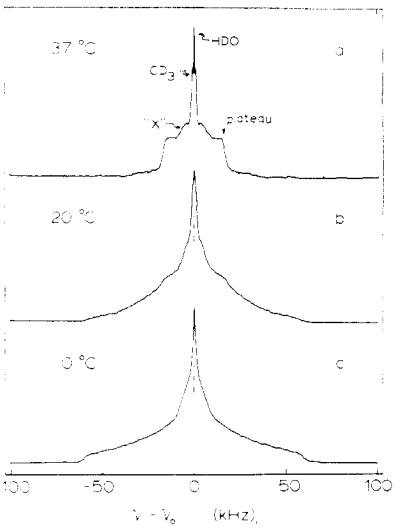

FIGURE 1: Deuterium NMR spectra at 34.4 MHz of outer membranes of *E. coli* grown on a medium containing perdeuterated palmitic acid. These spectra are the result of ~40 000 scans taken at a rate of 4 scans/s. (a) The spectrum at 37 °C showing the sharp HDO peak, the methyl group powder pattern, the distinctive feature X, and the plateau edge; (b) the spectrum at 20 °C; and (c) the spectrum at 0 °C; note the large intensity out to ±63 kHz.

Fatty acid compositions of the lipids of the fresh cells and of the membrane preparations are given in Table II. The lipid fatty acid composition of the cells grown with perdeuterated palmitic acid for 9 generations is comparable to that reported for the phospholipids of this strain grown in a slightly different medium (Baldassare et al., 1976). The perdeuterated palmitic acid content of the separated membranes is ~5% higher when oleic acid was not present in the growth medium. The increase in 18:1 acid in the membranes grown with both oleic and perdeuterated palmitic in the medium was accompanied by a decrease in the percentage of 16:1 palmitoleic acid present. In each of the two preparations, the outer membrane contained more saturated fatty acids and the cytoplasmic membrane more unsaturated fatty acids.

^{2}H NMR Spectra of Outer and Cytoplasmic Membranes. The spectra in Figure 1 are of the outer membranes of *E. coli* grown in the medium containing perdeuterated palmitic acid but *no* unsaturated fatty acids. The spectrum at 37 °C, shown

Table II: Fatty Acid Composition of the Phospholipids of Cells and Membranes of *E. coli* Strain L51 Grown with 50 μg/mL Perdeuterated Palmitic Acid Supplement[a]

sample	addtl FA suppl	FA, % satd 14	satd [^{2}H]16 + 16	satd 18	monounsatd 14:1	monounsatd 16:1	monounsatd 18:1	total satd/ total unsatd[e]
intact cells[b]		8	50 + 9			24	3	67:27
cytoplasmic membr[c]		2	37 + 18	3		28	12	60:40
outer membr[c]		4	39 + 22	4		21	8	69:29
cytoplasmic membr[d]	50 μg/mL	6	32 + 15	2		19	23	55:42
outer membr[d]	oleic acid	5	35 + 20	5		12	20	65:32

[a] Abbreviations: FA, fatty acid; suppl, supplement; satd, saturated; membr, membrane; addtl, additional. [b] Cells were grown for nine generations in basic growth medium. [c] Cells were grown for three generations in basic growth medium + 0.05% v/v PPBE. [d] Cells were grown for three generations in basic growth medium + 0.05% v/v PPBE + 0.4% casein hydrolysate. [e] Totals include only those fatty acids having 14, 16, or 18 carbons.

in Figure 1a, exhibits the characteristic features of a liquid-crystalline sample. The strong, sharp edge indicates that there is a well-defined plateau. The quadrupolar splitting of this edge gives the value of $(S_{CD})_{plateau} \approx 0.26$, which is typical of phospholipid lamellar phases just above the phase transition (Stockton et al., 1977; Davis, 1979). The splitting of the methyl group, $\delta\nu \approx 2.2$ kHz, gives $(S_{CD})_{methyl} \approx 0.017$, which is also typical of a lamellar liquid-crystalline phase. The sharp central peak in this and all of the other spectra is from the natural abundance of HDO in the water. There is an additional feature in this spectrum which did not appear in the spectra of perdeuterated DPPC (Davis et al., 1976) or *A. laidlawii* membranes (Stockton et al., 1977). This feature, which is labeled "X" in Figure 1a, comprises less than 15% of the intensity of the spectrum.

As the temperature is lowered from 37 °C, there is a gradual increase in intensity at higher frequencies, corresponding to larger quadrupolar splittings, associated with the appearance of gel phase regions in the sample. The spectrum in Figure 1b, obtained at 20 °C, is characteristic of this mixed phase region. There is intensity out to ±63 kHz, as expected for the gel phase, but the small shoulder occurring at ~25 kHz, in this spectrum and in those at higher temperatures, indicates that there is still a significant fraction of liquid-crystalline phase. As the temperature is lowered further, the component of the spectrum associated with the fluid phase continues to decrease in intensity. At 0 °C, the spectrum is that shown in Figure 1c. There is no clear indication of a fluid component and there is a sharp cut-off in intensity at ±63 kHz. The model of the gel phase where the acyl chains are in the all-trans conformation predicts that the spectrum should have the classic powder pattern line shape with a quadrupolar splitting of 63 kHz (±31.5 kHz), corresponding to $S_{CD} = 0.5$ if the molecules are all rapidly rotating about their long molecular axis (since in this model all methylene groups are equivalent except perhaps those in the α or first position on the chains). The spectrum in Figure 1c clearly does not support this model. The large intensity at ±63 kHz indicates that there is a large fraction of immobile (nonrotating) phospholipid and the shape of the spectrum suggests that there is a broad continuous distribution of quadrupolar splittings. This spectrum, apart from the central peak due largely to the natural abundance deuterium in the water, is very similar to the spectra observed in DPPC-d_{62} below the phase transition (Davis, 1979).

Figure 2 shows three spectra of the cytoplasmic membrane taken at temperatures similar to those of the spectra in Figure 1. At 34 °C the spectrum is narrow with a sharp edge at ca. ±12.6 kHz, giving $(S_{CD})_{plateau} \approx 0.20$, a value which is slightly smaller than that of the outer membrane, suggesting that the inner membrane is more fluid than the outer. The methyl group has a splitting of ≈1.6 kHz for this spectrum. This small splitting is not very well resolved because it is masked by the central HDO peak. The distinctive feature X mentioned earlier is also present in this spectrum. This feature has roughly the same size and shape in this spectrum that it has in the spectrum of Figure 1a. When superimposed on the characteristic bilayer component of these spectra, the smaller plateau splitting of the cytoplasmic membrane results in the slightly different shape of the cytoplasmic and outer membrane spectra.

At 19 °C, the spectrum of the cytoplasmic membrane, Figure 2b, clearly shows the presence of both gel and liquid-crystalline components. There is intensity out to ±63 kHz, and the liquid-crystalline plateau edge is still strong. This spectrum contains a much larger fluid component than the

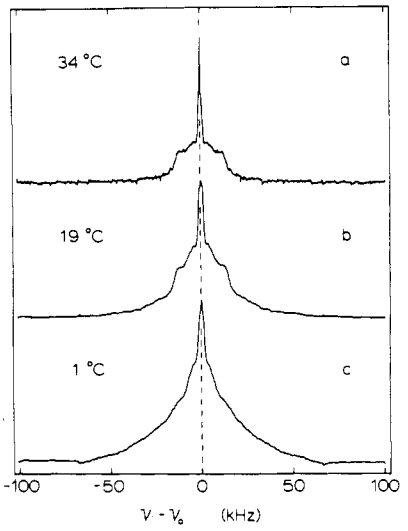

FIGURE 2: Deuterium NMR spectra at 34.4 MHz of the cytoplasmic membranes of *E. coli* grown on a medium containing perdeuterated palmitic acid. The repetition rate is 4 scans/s. (a) The spectrum at 34 °C shows the sharp HDO peak, the methyl group signal, the distinctive feature X, and the plateau edge, ~40 000 scans; (b) the spectrum at 19 °C possesses a large fraction of fluid component, ~60 000 scans; (c) the spectrum at 0 °C, ~70 000 scans.

spectrum of the outer membrane at 20 °C. In fact, the spectrum at 0 °C in Figure 2c looks very similar to the outer membrane spectrum at 20 °C, Figure 1b.

The spectra indicate clearly that, at and above about 37 °C, both the cytoplasmic and outer membranes are in the fluid phase. From the spectra in Figure 1a and 2a, we can estimate that at least 95% of the labeled phospholipids are in a fluid state (if the distinctive feature X is assumed to be associated with a fluid phase, see below). At 0 °C, the spectrum for the cytoplasmic membrane, Figure 2c, indicates that there is still a small amount of fluid lipid. The spectrum of the outer membrane at 0 °C is very similar to the gel phase spectrum of DPPC-d_{62} (Davis, 1979); however, because of the nature of this spectrum, we cannot place an accurate lower limit on the amount of fluid phase present.

The ^{2}H NMR spectra of membranes of *E. coli* grown in the medium containing oleic acid as well as perdeuterated palmitic acid have also been recorded as a function of temperature. The spectra were found to be qualitatively similar to those obtained from the membranes of cells grown in the absence of unsaturated fatty acids. An important difference between the two sets of membranes is that those from cells grown in the presence of oleic acid were fluid at much lower temperatures. The outer membrane spectrum at 22 °C, Figure 3a, and the cytoplasmic membrane spectrum at 19 °C, Figure 3b, are both nearly typical fluid phase spectra, although there is a small amount (10–15%) of gel phase component to these spectra. The spectrum in Figure 3a has $(S_{CD})_{plateau} \approx 0.28$ and that in Figure 3b has $(S_{CD})_{plateau} \approx 0.22$.

^{2}H NMR Spectra of the Outer Membrane after Lyophilization. After the measurements on the outer membranes of the cells grown in the absence of unsaturated fatty acids were completed, the sample was lyophilized and deuterium spectra were taken at 37, 20, and 0 °C after resuspending the dried membranes in water. Each of these spectra was visually identical (within the signal to noise ratio) with the earlier spectrum of the nonlyophilized sample taken at the same temperature, Figures 1a–c. At 37 °C, the first eight moments (which are sensitive functions of the shape of the spectrum) of the spectra taken before and after lyophilization agreed to within 2%. At 20 °C, the agreement of the first eight moments

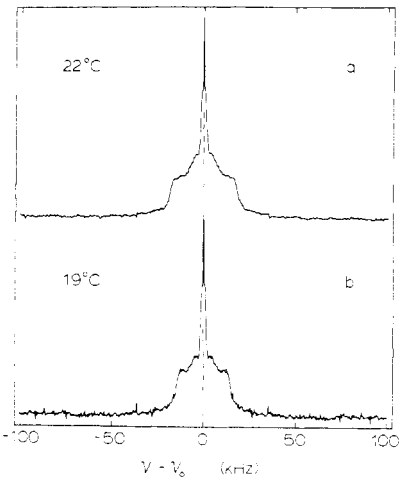

FIGURE 3: Deuterium NMR spectra at 34.4 MHz of the membranes of *E. coli* grown on a medium containing oleic acid as well as perdeuterated palmitic acid. The repetition rate is 4 scans/s. (a) The outer membrane at 22 °C, ~20 000 scans; (b) the cytoplasmic membrane at 19 °C, ~40 000 scans.

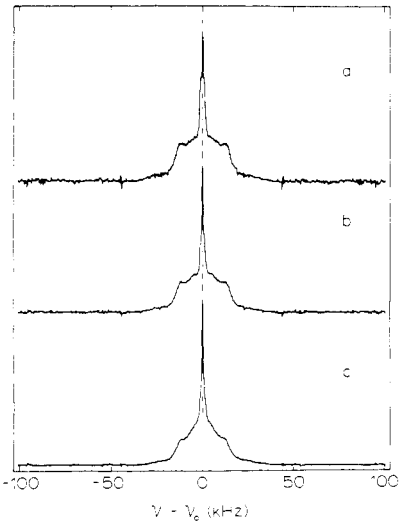

FIGURE 4: Deuterium NMR spectra at 34.4 MHz at 37 °C of intact cells of *E. coli* grown on a medium containing perdeuterated palmitic acid. The repetition rate is 4 scans/s. (a) Spectrum recorded from 2.5 to 6.0 h after preparation of the cells, ~23 000 scans; (b) 6.5–8.5 h after preparation, ~29 000 scans; (c) 8.5–18 h after preparation, ~131 000 scans.

was within 5%, and at 0 °C the first four moments agreed within 5%. This remarkable agreement between spectra taken before and after lyophilization shows clearly that lyophilization followed by resuspension in H_2O does not alter the *physical* state of the lipids of *E. coli* membranes as determined by deuterium magnetic resonance. As ^2H NMR has proven to be one of the most accurate and sensitive techniques of monitoring the physical state of the bilayer, this result indicates that lyophilization of the sample can be used in membrane studies where the techniques are sensitive to the physical state of the bilayer.

^2H NMR Spectra of Intact Cells and Cell Envelopes. Three ^2H NMR spectra at 37 °C of intact cells of *E. coli* grown in the medium containing perdeuterated palmitic acid but no unsaturated fatty acids are shown in Figure 4. These three spectra, recorded over times t = 2.5–6.0, 6.5–8.5, and 8.5–18 h after preparation of the cells, illustrate the degradation of the sample with time. Each of these spectra exhibits the same basic spectral features as the spectra in Figures 1a and 2a, as expected. An interesting aspect of these spectra is that the

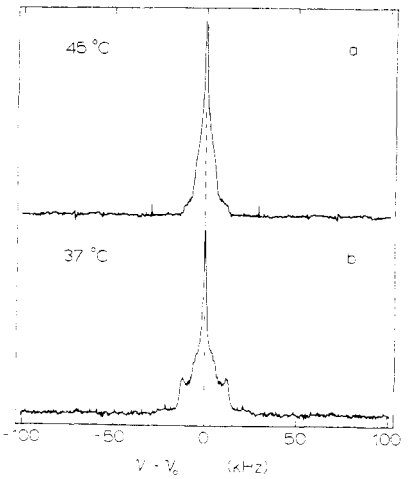

FIGURE 5: Deuterium NMR spectra at 34.4 MHz of lipids extracted from the cytoplasmic membranes of *E. coli* grown on a medium containing perdeuterated palmitic acid. The repetition rate is 4 scans/s. (a) The spectrum at 45 °C, 120 000 scans; (b) the spectrum at 37 °C, 120 000 scans.

feature X shows an increase in relative intensity with time. Also, repetition of the intact cell experiments revealed that this central feature was smaller when the time between harvesting the cells and their insertion into the NMR spectrometer was shorter. These observations suggest that feature X is associated with the degradation that occurs when the cells are densely packed without nutrients at physiological temperatures.

Spectra of intact cells were also recorded at lower temperatures and were similar to the lower temperature spectra of Figures 1 and 2. Because of the rapid degradation of the sample, a detailed study of the temperature dependence of the spectra of intact cells could not be accomplished.

The cell envelopes, which are unseparated cytoplasmic and outer membranes, gave ^2H NMR spectra qualitatively similar to those obtained with the intact cells and the separated membranes. There was a well-defined plateau edge occurring at ~30 kHz at 37 °C corresponding to $(S_{CD})_{plateau} \approx 0.24$, in good agreement with the value of 0.23 for intact cells.

^2H NMR Spectra of Lipids Extracted from the Cytoplasmic Membrane of E. coli. Figure 5 shows two deuterium spectra of the lipids extracted from the cytoplasmic membrane of cells grown on the medium containing oleic acid as well as perdeuterated palmitic acid. The spectrum at 37 °C (Figure 5b) looks quite similar to that of the corresponding cytoplasmic membrane at the same temperature. The plateau edge is well defined and gives the values of $(S_{CD})_{plateau} \approx 0.21$, identical within the experimental error to the value of 0.20 obtained for the membranes. The plateau seems to be more sharply defined in the extracted lipids and there is also a clearer separation between feature X and the rest of the spectrum. The spectrum of the extracted lipids taken at 45 °C (Figure 5a) appears to consist of only this central feature. The spectrum of lipids extracted from the outer membrane at 45 °C also consists only of feature X (data not shown). If we measure an edge order parameter for this spectrum we find $(S_{CD})_{edge} \approx 0.10$, approximately equal to half the value of $(S_{CD})_{plateau}$ at 37 °C. A possible explanation for this peculiar spectrum is that the extracted lipids have undergone a transition from a lamellar to an hexagonal phase. The extra motional averaging of the quadrupolar interaction caused by the diffusion of the lipid molecules around the circumference of the hexagonal rods can result in a decrease in the measured quadrupolar splittings by a factor of two (Mely et al., 1976).

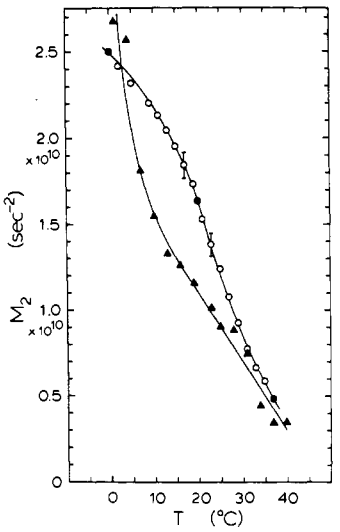

FIGURE 6: Second moments vs. temperature of the spectra of outer (circles) and cytoplasmic (triangles) membranes of *E. coli* grown on a medium containing perdeuterated palmitic acid. The data shown as open circles are for spectra obtained using 9-μs pulses and have been adjusted to agree with the data shown as solid circles which are for spectra obtained with 4.5-μs pulses. The error bars show the estimated error of ±5% not including the systematic error due to finite pulse width (significant at low temperature).

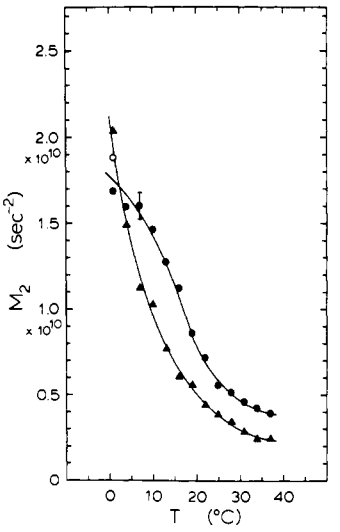

FIGURE 7: Second moments vs. temperature of the spectra of outer (circles) and cytoplasmic (triangles) membranes of *E. coli* grown on a medium containing oleic acid as well as perdeuterated palmitic acid. The open circle gives the second moment of the outer membrane spectrum at 1 °C obtained by using 2.25-μs pulses; all other values are for spectra obtained by using 4.5-μs pulses. The error bars show the estimated error of ±5%.

The distinctive shape of this spectrum is what one would expect if the splitting of each powder pattern component of the spectrum was decreased by a factor of two, keeping the width of each component constant. This would result in the build-up of intensity near the center of the spectrum.

It appears from these studies that the existence of this feature X and its time-dependent increase in the spectra of intact cells is related to membrane disruption and the possible formation of an hexagonal phase by the phospholipids in the membrane fragments.

Moments of the ^2H NMR Spectra. The ^2H NMR spectra discussed in this section have enabled us to make a number of qualitative statements about the physical states of the *E. coli* membranes. In particular we know that, at higher temperatures (e.g., at 37 °C), both the outer and cytoplasmic membranes are in a fluid phase and that, as the temperature is lowered, there is a gradual increase in the amount of gel phase present until at a temperature of 0 °C essentially all of the outer membrane and a large fraction of the cytoplasmic membrane are in the gel phase. The use of samples from cells grown on specifically deuterium-labeled fatty acids would in principle allow a detailed, quantitative interpretation of the spectra reported here. However, even when the ^2H NMR samples are from cells grown on perdeuterated fatty acids, the method of moments described earlier can be used to make a significant quantitative comparison of the cytoplasmic and outer membranes of *E. coli*.

The values of M_n are easily calculated from ^2H NMR spectra such as those shown in Figures 1–5. We will here present only the results of the evaluation of the *second moment*, M_2, for the four membrane samples: the cytoplasmic and outer membranes of cells (1) grown on the medium containing perdeuterated palmitic acid and (2) grown on the medium containing both oleic acid and perdeuterated palmitic acid.

As discussed earlier (eq 2 and 3), the second moment of a ^2H NMR spectrum is directly related to the mean square order parameter $\langle S_{CD}^2 \rangle$. The observed increase in the mean order parameter by more than a factor of two on decreasing the temperature through the fluid to gel transition is reflected in a large increase in the second moment. The transition from the liquid-crystalline to gel phase in DPPC-d_{62} model membranes takes place over a temperature range of about 1 °C and leads to a change in M_2 of the ^2H NMR spectrum by about a factor of five (Davis, 1979). Figure 6 shows the variation of the second moment, M_2, of the cytoplasmic and outer membranes of cells grown in the medium containing perdeuterated palmitic acid. As in the model membrane, there is a change in M_2 of both membrane samples by about a factor of five. For *E. coli* membranes, however, the change occurs over the entire temperature range, from 0 to 37 °C. This result is in agreement with earlier studies which showed that these membranes undergo a very broad phase transition (Overath et al., 1975). Perhaps the most striking feature of these data is the fact that above 5 °C the second moment of the cytoplasmic membrane is systematically smaller than that of the outer membrane.

One way of describing this systematic difference is to note that, if the M_2 vs. temperature data for the cytoplasmic membrane are displaced by about +7 °C, they overlap those of the outer membrane over a substantial fraction of the temperature range studied. We suggest, therefore, that the phase transition of the cytoplasmic membrane occurs over a region approximately 7 °C lower in temperature than that of the outer membrane. This method of characterizing the difference between the orientational order of the two membranes is not necessarily unique since the order may depend on molecular composition as well as on the relative amounts of gel and fluid components. As pointed out in our earlier discussion of the form of the ^2H NMR spectra, however, the cytoplasmic membrane has at any temperature a greater fraction of its intensity associated with the characteristic liquid-crystalline feature. We shall, therefore, continue to compare the ^2H NMR measurements on the cytoplasmic and outer membranes in terms of the apparent temperature difference of their transition regions.

The second moment data, Figure 7, for membranes of cells grown on the medium containing both oleic and perdeuterated palmitic acid shows the same general behavior, an increase in M_2 by about a factor of five over the entire temperature range, with the second moment of the cytoplasmic membrane systematically smaller than that of the outer membrane. In

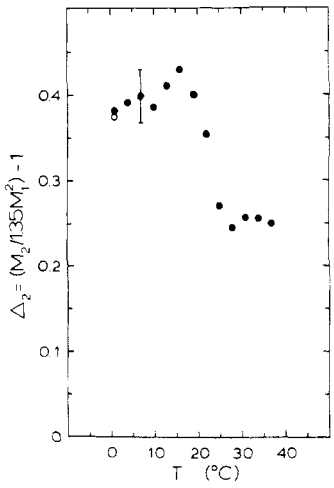

FIGURE 8: The parameter Δ_2 vs. temperature of the spectra of the outer membranes of *E. coli* grown on a medium containing oleic acid as well as perdeuterated palmitic acid. The open circle gives the value of Δ_2 for the spectrum at 1 °C obtained by using 2.25-μs pulses; all other values are for spectra obtained by using 4.5-μs pulses. The error bars show the estimated error of ±7.5%.

this case, not only is the phase transition of the cytoplasmic membrane about 7 °C lower than that of the outer membrane, but the transitions of both membranes are at lower temperatures than those of the membranes of Figure 6.

The parameter Δ_2 defined by eq 4 in terms of the first two moments of the spectrum gives the mean square width of the distribution of order parameters. This parameter is sensitive to the coexistence of phases with very different mean order parameters, such as occurs for the gel and fluid phases. In DPPC-d_{62}, Δ_2 goes through a strong maximum in the phase transition region (Davis, 1979). Figure 8 is a plot of Δ_2 vs. temperature for the outer membranes of the cells grown with the mixture of oleic and perdeuterated palmitic acids. There is a substantial increase in Δ_2 below ~25 °C and a maximum at ~16 °C. The value of Δ_2 above 30 °C is roughly constant at ~0.25, which is much larger than the value of 0.09 for liquid-crystalline DPPC-d_{62} (Davis, 1979). Similarly, the values of Δ_2 at low temperatures for this sample as well as for the other *E. coli* membrane samples are much higher than those found in the gel phase of the model system. The heterogeneity of the phospholipids in the *E. coli* membranes as well as the presence of membrane-bound proteins and lipopolysaccharide are possible explanations of this broader distribution of S_{CD}.

Concluding Remarks

The deuterium spectra of intact cells, cell envelopes, and the separated cytoplasmic and outer membranes of *E. coli* grown in media supplemented with perdeuterated palmitic acid exhibit features which may be called typical of the phospholipid bilayer. The spectra are similar to those of DPPC-d_{62} model membranes (Davis, 1979) and of *Acholeplasma laidlawii* grown in media supplemented with perdeuterated palmitic acid (Stockton et al., 1977).

The primary feature of the spectra at high temperature (≥37 °C) of all of the samples reported here is the sharp edge due to the well-defined order parameter plateau characteristic of the phospholipid bilayer's fluid phase. It appears, as viewed by ^{2}H NMR, that in the biological membranes studied here all of the phospholipid molecules (except for the 10–15% contributing to the "X" feature of the spectrum) are equivalent at these high temperatures, even though the presence of membrane-bound proteins ensures the existence of inequivalent phospholipid sites. It is very unlikely that the local orientational order should be independent of the distance from protein molecules. However, distinct ^{2}H NMR spectra from deuterium nuclei on phospholipid molecules at inequivalent sites would only be observed if the difference between the quadrupolar splittings, which are a measure of the degree of order of those sites, was much greater than the frequency with which phospholipid molecules exchange between the inequivalent sites. Our results at high temperatures, even though they have been obtained with *perdeuterated* samples, indicate that, if the *variations* in quadrupolar splittings among inequivalent sites are of the same order as the splittings ($\delta\nu$), then such exchange processes must take place within a time much less than $1/(2\pi\delta\nu)$. This conclusion is similar to that reached by several research groups who have conducted ^{2}H NMR studies on reconstituted membranes (Seelig & Seelig, 1979; Oldfield et al., 1978; F. W. Dahlquist, M. Paddy, J. H. Davis, and M. Bloom, unpublished results[3]) and is the conclusion which must also be drawn from the high temperature ^{2}H NMR measurements previously reported on *Acholeplasma laidlawii* membranes (Stockton et al., 1977).

The observation that the orientational order parameter of the plateau of the saturated hydrocarbon chains in the cytoplasmic and outer membranes at high temperatures is quite close to that found in the liquid-crystalline phase of model membranes and in the extracted lipids is quite remarkable when one considers that the phospholipids in these systems must spend an appreciable fraction of the time in close contact with proteins. For example, the lipid to protein ratio of the outer membrane (Osborn et al., 1972) suggests that the outer membrane phospholipids must be in direct contact with proteins about half of the time, assuming rapid exchange. Therefore, at high temperatures the interaction of the membrane phospholipids with the protein surfaces, which are expected to be relatively rigid, must result in local orientational order parameters which are not *very* different from values obtained in the absence of proteins.

As the temperature is decreased below 37 °C, the characteristic gel spectrum appears superimposed on the liquid-crystalline spectrum for each of the membrane samples studied. It is obvious from these spectra that large gel and liquid-crystalline regions coexist in these systems over a wide range of temperatures, the fraction of phospholipids in the liquid-crystalline state decreasing steadily as the temperature is lowered. Visual inspection of the ^{2}H NMR spectra and the second moment measurements both lead to the following conclusions.

(1) The cytoplasmic membrane of a given bacterial preparation was generally more fluid at a given temperature than the outer membrane. The words "more fluid" here have two implications: a greater degree of orientational disorder as determined from second moment measurements and a larger proportion of the spectral intensity being associated with the characteristic liquid-crystalline signal according to visual inspection of the spectra.

(2) Inclusion of oleic acid in the growth medium produced a marked increase in fluidity at all temperatures. In fact, whereas the membranes from cells grown only with the deuterated palmitate supplement exhibited no detectable

[3] Distinct spectra corresponding to boundary and bulk lipids in a reconstituted membrane have been reported (Dahlquist et al., 1977), but subsequent studies (F. W. Dahlquist, M. Paddy, J. H. Davis, and M. Bloom, unpublished results) with similarly prepared samples indicate only one liquid-crystalline type of ^{2}H NMR spectrum in agreement with the observations of Seelig & Seelig (1979) and Oldfield et al. (1978).

liquid-crystalline features at 0 °C, a substantial liquid-crystalline signal was still observed at this temperature in samples grown with oleic acid in addition to the (deuterated) palmitic acid. In the samples studied, the effect of the oleic acid supplement was to increase only slightly the ratio of the total number of unsaturated to saturated hydrocarbon chains, but it did cause an increase in the fraction of 18:1 chains at the expense of the 16:1 chains. The resulting decrease in the temperature of the transition region can be compared with that observed via calorimetry by other workers in two similarly related lipid samples (compare the thermal behavior of samples 4 and 2 of Table I in the paper by Baldassare et al., 1976).

(3) The variation of orientational order over the transition region, as measured from the second moments, was roughly the same for both the cytoplasmic and outer membranes as for DPPC model membranes. This result suggests that the phospholipids in the outer membrane samples probably have a bilayer structure in spite of the low lipid:protein ratio and the small proportion of chains in the outer membrane phospholipids "taking part in the transition" according to X-ray diffraction experiments (25–40%) (Overath et al., 1975).

The relationship between the liquid crystalline transition as viewed by the ^2H NMR and X-ray diffraction experiments deserves some further discussion. As described above, the magnetic resonance experiments show distinctly that almost all of the phospholipids exhibit orientational order characteristic of the liquid-crystalline phase at high temperatures and that the liquid-crystalline spectrum disappears gradually as the temperature is lowered through the transition region. Thus the ^2H NMR experiments suggest that almost all of the saturated chains of the phospholipid molecules take part in the order–disorder transition. The X-ray diffraction experiments carried out on similar, though not identical, bacterial membranes (Overath et al., 1975) suggest that only a fraction of the chains take part in the transition: 60–80% for the cytoplasmic membranes and 25–40% for the outer membranes. These results are *not* contradictory. Rather, the two techniques are complementary to each other since they are sensitive to quite different physical properties. Care must be taken in comparing results obtained with the two techniques.

The ^2H NMR spectrum is a superposition of spectra from *all* the deuterium nuclei in the sample. Each nucleus contributes exactly the same integrated intensity to the spectrum independently of whether it is located in a region of large or small long range order. Furthermore, the orientational order parameters which determine the shape and width of the ^2H NMR spectrum are only sensitive to the spatial distribution and motions of charges in the immediate vicinity of the deuterium nuclei. Thus the ^2H NMR experiment provides an accurate measure of the distribution of orientational order parameters for the entire system, but provides no direct information on the spatial distribution of the nuclei associated with the different order parameters.

By contrast, X-ray diffraction provides structural information through the analysis of coherently scattered X-rays. The fraction of saturated hydrocarbon chains which are "ordered" according to X-ray diffraction measurements on biological membranes is obtained from the integrated intensity of the $(4.2\ \text{Å})^{-1}$ Bragg reflection (Schecter et al., 1974; Overath et al., 1975). The intensity of this peak is sensitive not only to the orientational order of the hydrocarbon chains, but also to the average size of domains of phospholipid molecules having their long axes on a regular two dimensional lattice. If the domains are too small, the Bragg peak is broadened to such an extent that it cannot be distinguished from the background of incoherently scattered X-rays. The small fraction of ordered chains measured by X-ray diffraction relative to those which are orientationally ordered according to magnetic resonance measurements implies, therefore, that a large fraction of the oriented phospholipid molecules at low temperatures are in small domains having no more than a few hundred molecules. This conclusion is not at all surprising in view of the large number of proteins in these systems. The fact that the outer membrane has a much smaller fraction of ordered chains than the cytoplasmic membrane is probably a manifestation not of orientational ordering but of the smaller average domain size associated with a larger protein concentration.

Further studies using both phosphorus NMR and deuterium NMR on specifically deuterated membranes and lipid extracts are underway. It will be of interest to compare nuclear magnetic resonance measurements of the type described here more closely with X-ray diffraction and other physical measurements on biological membranes and to develop more precise relationships between the different measurements.

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Thermal Behavior of Synthetic Sphingomyelin–Cholesterol Dispersions†

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ABSTRACT: The thermotropic behavior of aqueous dispersions of palmitoylsphingomyelin–cholesterol and lignocerylsphingomyelin–cholesterol mixtures has been examined by high-sensitivity differential scanning calorimetry. When less than 25 mol % cholesterol is mixed with either sphingomyelin, the calorimetric endotherm is composed of a sharp and a broad component. The sharp-component enthalpy change decreases as the mole percent cholesterol increases with the extrapolated zero enthalpy point being 25 to 30 mol %. With palmitoylsphingomyelin, the temperature of maximum heat capacity of the sharp component decreases monotonically with increasing cholesterol content, while the lignocerylsphingomyelin sharp-component maximum remains constant until more than 20 mol % sterol is present. The broad-component enthalpy change maximizes at 3–4 kcal/mol between 10 and 20 mol % cholesterol and decreases as the ratio of cholesterol is increased or decreased from this range for both sphingomyelins. The results are compared with those from a previous study on dipalmitoylphosphatidylcholine–cholesterol mixtures and are interpreted as evidence for the coexistence of cholesterol-rich and cholesterol-poor phases.

Sphingomyelin is a primary constituent of many mammalian membranes, comprising up to 60 mol % of the total phospholipid in some tissues (Rouser & Solomon, 1969; Broekhuyse, 1969). The relative concentration of this lipid is a parameter of some physical significance since membrane permeability and osmotic fragility are highly correlated with sphingomyelin content (Hertz & Barenholz, 1975; Kirk, 1977; Borochov et al., 1977). This lipid also appears to be the binding site for acetylcholinesterase (Watkins et al., 1977) and for certain cytolytic toxins (Linder et al., 1977). In addition, sphingomyelin is intimately involved with the aging process and a number of pathological conditions. For example, the sphingomyelin content of human aorta and lens tissue increases several fold over a normal lifespan (Rouser & Solomon, 1969; Broekhuyse, 1969), while proper fetal lung development is characterized by a relative decrease in this lipid during the last few weeks of gestation (Gluck et al., 1971). Portman (1970) has associated an elevation of sphingomyelin concentration with the initiation of atherosclerosis in squirrel monkeys, and Hughes (1972) has reported that the amount of this lipid is elevated in dystrophic muscle tissue. For these reasons, the study of the interaction of sphingomyelin with other lipids is of clinical significance as well as being of interest to researchers concerned with fundamental questions of membrane structure.

Of particular interest is the characterization of sphingomyelin–cholesterol mixtures. Cholesterol is often found in significant quantities in membranes which contain a relatively high proportion of sphingomyelin (Patton, 1970), and it has been suggested that these two lipids form a complex (Patton, 1970; Vandenheuvel, 1963). Recently, Demel et al. (1977)

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