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Quantitative Study of the Fluidity of *Escherichia coli* Membranes Using Deuterium Magnetic Resonance†

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ABSTRACT: Specifically deuterated palmitic acid was incorporated into the membrane phospholipids of the L51 strain of *Escherichia coli*. The cytoplasmic and outer membranes were separated by using standard techniques and studied by deuterium nuclear magnetic resonance between 0 and 40 °C. Distinctive liquid-crystalline and gel spectra were observed to coexist over a wide temperature range. The relative intensities of these spectra provided a direct measure of the fraction of the deuterium-labeled phospholipids in the fluid state as a function of temperature. Above 37 °C, the amount of immobilized or gel-phase phospholipid is estimated to be less than 3% of the total phospholipid. The gel to liquid-crystalline transition region for the outer membrane was shifted upwards by ~7 °C relative to that of the cytoplasmic membrane, in agreement with previous studies [Davis, J. H., Nichol, C. P., Weeks, G., & Bloom, M. (1979) *Biochemistry 18*, 2103]. The orientational order in the fluid phase of both membranes decreased gradually with increasing temperature and was greater in the outer membrane than in the cytoplasmic membrane. The orientational order of the gel-phase component was the same for both membranes, within an experimental uncertainty of 10%, and was independent of temperature from 0 to 30 °C for the outer membrane and from 10 to 30 °C for the cytoplasmic membrane.

The membrane phospholipids of *Escherichia coli* undergo a broad gel to liquid-crystalline phase transition which has been studied by a variety of techniques, including differential scanning calorimetry (Baldassare et al., 1976; Jackson & Sturtevant, 1977; Jackson & Cronan, 1978), X-ray diffraction (Overath et al., 1975; Schechter et al., 1974; Linden et al., 1977; Harder & Banaszak, 1979), electron spin resonance (Rottem & Leive, 1977; Linden et al., 1973; Sackmann et al., 1973), and fluorescence (Overath & Träuble, 1973; Cheng et al., 1974; Overath et al., 1975). More recently, this transition has been studied by deuterium nuclear magnetic resonance (^{2}H NMR[1]) (Davis et al., 1979; Kang et al., 1979b).

Cronan & Gelmann (1975) and Cronan (1978) have reviewed the numerous studies on the relationship between the physical state of the membranes and their physiological functions. In a recent calorimetric study, Jackson & Cronan (1978) reported that, for wild type *E. coli*, the entire phase-transition region of the extracted lipids is well below the growth temperature (whether the cells were grown at 37 °C or at 25 °C). They also demonstrated by calorimetry on the extracted lipids that certain *E. coli* mutants can grow (at a reduced rate) at a temperature where more than half of the lipids are in the gel phase.

There is some difficulty in estimating the relative fractions of fluid- and gel-phase lipid from calorimetric data in whole membranes because nonlipid components contribute to the thermal properties. The fractions of fluid- and gel-phase lipid in *E. coli* membranes have been measured as a function of temperature by X-ray diffraction (Overath et al., 1975). However, when this method is used, the intensity of the high-angle Bragg peak (at 4.2 Å) of the gel-phase lipids depends on the average size of the gel-phase domains (long-range order) as well as on the orientational (short-range) order of the hydrocarbon chains. Lipid molecules in small spatially ordered domains will not contribute to this X-ray diffraction peak.

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[1] Abbreviations used: ^{2}H NMR, deuterium nuclear magnetic resonance; GLC, gas–liquid chromatography; DEGS, diethylene glycol succinate; KDO, 2-keto-3-deoxyoctonate; LPS, lipopolysaccharide; DPPC, dipalmitoylphosphatidylcholine.

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In ^2H NMR spectra of membranes, the fluid- and gel-phase regions each contribute characteristic, readily distinguishable components (a narrow fluid-phase component and a broad gel-phase component[2]) to the ^2H NMR spectrum. Since each deuterium-labeled phospholipid molecule contributes the same integrated intensity to the ^2H NMR spectrum, measurement of the areas of the two components of the spectrum quantitatively determines the relative fractions of fluid- and gel-phase lipid.

In our previous ^2H NMR study on *E. coli* grown with palmitic-d_{31} acid, we demonstrated that the temperature range of the phase transition of the outer membrane was higher than that of the cytoplasmic membrane. This same result was obtained when the growth medium contained oleic acid as well as palmitic-d_{31} acid. In addition, this increased percentage of oleic acid in the membrane lipids produced a substantial decrease in the phase-transition temperature for both the cytoplasmic and outer membranes (Davis et al., 1979). While the reported differences in the fluidity of these membranes could be correlated with the difference in phospholipid acyl chain composition, the influence of other factors on the fluidity of the membranes could not be established. Specifically, it was not clear whether the higher concentration of protein or the presence of large amounts of lipopolysaccharide (LPS) in the outer membrane contributed to that membrane's lower fluidity. In the present study we show that, at a given temperature, the orientational order of the fluid-phase component of the outer membrane is higher than that of the fluid-phase component of the cytoplasmic membrane and there is a larger fraction of fluid-phase lipid in the cytoplasmic membrane than in the outer membrane. In addition, we show that, over most of the temperature range in which the gel and fluid regions coexist, the mean values of the gel-phase orientational order are equal to each other and independent of temperature, within experimental error.

Materials and Methods

Palmitic-*13,13*-d_2 acid was the gift of Drs. I. C. P. Smith and A. P. Tulloch.

E. coli Strain and Growth Conditions. *E. coli* strain L51 (the gift of Dr. D. F. Silbert) incorporates exogenous fatty acid into its membrane lipids and is defective in fatty acid oxidation (Silbert et al., 1973a). The basic growth medium, described previously (Davis et al., 1979), containing 50 μg/mL palmitic-*13,13*-d_2 acid was supplemented with 0.05 v/v proteose peptone–beef extract,[3] 50 μg/mL oleic acid, and 4 g/L casein hydrolysate (Nutritional Biochemical Corp., Cleveland, OH). Cells were grown in this medium overnight at 37 °C, and these adapted cells were used to inoculate fresh medium. The cells were grown at 37 °C with aeration for three generations and then harvested (at an absorbance at 600 nm of 0.88) at room temperature while still in the exponential phase of growth.

Preparation of Cytoplasmic and Outer Membranes. The preparation and characterization of the cytoplasmic and outer membranes by the method of Osborn & Munson (1974)[4] was as described previously (Davis et al., 1979). After the final centrifugation, each membrane pellet was transferred into a 7.5-mm diameter NMR tube.

Lipid Analysis. The lipids were extracted from the membranes by a modification (Ames, 1968) of the method of Bligh & Dyer (1959). The lipid extracts were saponified, and the methyl esters of the fatty acids were separated by gas–liquid chromatography (GLC) on a 12% DEGS column at 150 °C.

Other Analytical Procedures. Protein was determined by Lowry's method (Lowry et al., 1951) and 2-keto-3-deoxyoctonate (KDO) as described by Osborn et al. (1972). Succinate dehydrogenase was assayed at 30 °C as described previously (Lee et al., 1975).

Nuclear Magnetic Resonance Techniques. At the beginning of the series of measurements for each membrane preparation, the sample was allowed to equilibrate at 0 °C for 2 h. After this time the first ^2H NMR spectrum was recorded, the temperature was increased to the next higher temperature, and the sample was allowed to equilibrate for 40 min before the next spectrum was recorded. This procedure was then repeated for each temperature up to 42 °C.

The NMR methods and instrumentation used here were identical with those described previously (Davis et al., 1979). The ^2H NMR spectra were taken at 34.4 MHz, accumulating at a rate of 4 scans/s. When the radio-frequency excitation is applied at the central frequency of a symmetric spectrum, the out-of-phase component of the signal is zero. When the noise accumulated in that channel of our quadrature detector is zeroed, the resulting spectrum is perfectly symmetric since positive and negative frequencies are superimposed on one another. No filters were used during data acquisition, and no phase corrections were applied to the transformed spectra. Use of the Fourier transform quadrupolar echo technique (Davis et al., 1976) combined with the broad bandwidth of our detection system ensured that the spectra were nearly distortion free even for the broadest (~120 kHz) ^2H NMR lines recorded. In the severest cases, the spectral distortion due to finite pulse lengths can be corrected theoretically [M. Bloom, J. H. Davis, and M. I. Valic, unpublished work based on the treatment of spin 1 NMR systems by Vega & Pines (1977)].

An important aspect of our analysis of the ^2H NMR data is the use of the method of moments (Bloom et al., 1978; Davis et al., 1979). The ^2H NMR spectrum, $f(\omega)$, is symmetric about the angular Larmor frequency, ω_0, but it is useful to compute the moments, M_n, of the *half-spectrum* for $\omega \geq \omega_0$ or $\omega \leq \omega_0$. The moments are then defined by

$$M_n = \frac{\int_{\omega_0}^{\infty} (\omega - \omega_0)^n f(\omega)\, d\omega}{\int_{\omega_0}^{\infty} f(\omega)\, d\omega} \qquad (1)$$

The strength of the residual quadrupolar interaction is determined by an average over the fluctuations of the angle (θ) between the C–D bond vector (the symmetry axis of the electric field gradient at the deuterium nucleus) and the symmetry axis of the motion (taken to be the normal to the bilayer). If we neglect all other contributions to the spectrum (e.g., dipolar broadening), the maximum information obtainable from ^2H NMR spectroscopy[5] is the probability distribution, $P(S)$, of the orientational order parameters, S, of the

[2] The term "gel phase component" is used loosely here to denote all broad spectral components.

[3] This was erroneously reported as 0.05% v/v in our previous paper (Davis et al., 1979).

[4] When Osborn's method is used and the murein layer is removed with lysozyme from the outer membrane, some of the lipopolysaccharide, expected to be in the outer leaflet, may be transposed to the inner leaflet of the outer membrane. This does not occur at 0 °C during the preparation of the membrane but can occur very rapidly in *Salmonella typhimurium* at 37 °C (Mühlradt & Golecki, 1975). It is therefore possible that the outer membrane preparations studied here may not have the same membrane asymmetry found in vivo.

[5] ^2H NMR measurements give information on the magnitude of S but not on its sign. Actually, S can vary between $S = -1/2$ and $S = +1$. Information on the sign of S can be obtained from anisotropic chemical-shift measurements [see, e.g., Seelig (1978)].

Table I: Fatty Acid Composition of the Lipids of Membranes of *E. coli* Strain L51 Grown with 50 μg/mL Oleic Acid and 50 μg/mL Palmitic-*13,13-d*$_2$ Acid

	fatty acid (%)							
	saturated			monounsaturated				
sample	14	16	18	14:1	16:1	18:1	total[a] satd (%)	total[a] unsatd (%)
cytoplasmic membrane	4	54		-	18	22	58	40
outer membrane	7	55	2	-	14	18	64	32

[a] Totals include only those fatty acids having 14, 16, and 18 carbons.

C–D bonds, where S, for a given C–D bond, is defined by (Seelig, 1977)

$$S = \langle \tfrac{1}{2}(3\cos^2\theta - 1)\rangle \quad (2)$$

and the angular brackets denote an average over all motions occurring at a rate greater than the inverse of the quadrupolar interaction strength.

In this situation we can relate the moments, S_n, of the order parameter distribution function $P(S)$, where

$$S_n = \int_0^1 S^n P(S)\, dS \quad (3)$$

to the moments, M_n, of the ^{2}H NMR spectra. For the nth moment

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

where $e^2qQ/\hbar = 2\pi \times (1.67 \times 10^5$ Hz) is the quadrupolar coupling constant for methyl or methylene groups (Seelig, 1977) and the coefficients $A_1 = 2/[3(3)^{1/2}]$, $A_2 = 1/5$, etc. are easily calculated (Bloom et al., 1978) from the expression for the spin 1 powder pattern line shape (Seelig, 1977). It should be emphasized that this calculation is straightforward. Equation 4 follows from eq 1–3 by using the results described below (eq 6 and 7) for a single value of S.

In this fashion, the first two moments of the spectrum, M_1 and M_2, determine the average order parameter, S_1, and its mean squared value, S_2. Once S_1 and S_2 are known, the fractional mean squared width, Δ_2, of the distribution of order parameters can be obtained.

$$\Delta_2 \equiv \frac{S_2 - S_1^2}{S_1^2} = \frac{M_2}{1.35M_1^2} - 1 \quad (5)$$

In a system having only a single value of S, a characteristic powder pattern spectrum is observed (Seelig, 1977). This spectrum has two sharp peaks separated by an amount, $\Delta\nu$, called the quadrupolar splitting. The value of this splitting provides a direct measure of the C–D bond order parameter, S, through the relation

$$2\pi\Delta\nu = \omega_Q = \frac{3}{4}\frac{e^2qQ}{\hbar}S \quad (6)$$

The moments of this spectrum

$$M_n = A_n\omega_Q^n \quad (7)$$

are completely determined by $\Delta\nu$ or, equivalently, by S. Such a spectrum is observed for the fluid phase of *E. coli* membranes of cells grown on media supplemented with palmitic acid deuterated in a single position.

In the temperature region over which the *E. coli* membranes are undergoing a fluid- to gel-phase transition, the sharp fluid-phase spectrum is superimposed on a much broader gel-phase spectrum. This coexistence of phases has also been studied by ^{2}H NMR of palmitate-*13,13-d*$_2$-enriched *Acholeplasma laidlawii* membranes (Smith et al., 1979). Assuming that the ^{2}H NMR spectrum in the transition region is a simple superposition of gel- and fluid-phase spectra, we may express the nth moment of the composite spectrum as

$$M_n = fM_n^{liq} + (1-f)M_n^{gel} = fA_n\omega_Q^n(\text{liq}) + (1-f)M_n^{gel} \quad (8)$$

where f is the fraction of deuterium-labeled phospholipid molecules in the fluid phase, M_n^{liq} and M_n^{gel} are the nth moments of the two components of the spectrum, and ω_Q(liq) is the quadrupolar splitting (in angular frequency units) of the fluid-phase component of the spectrum. The value of f is obtained directly from the spectrum as the ratio of the area of the liquid-crystalline component to the total area of the spectrum. Measurement of f, ω_Q, and M_n enables us to determine M_n^{gel} (from eq 8) at any temperature within the transition region.

Results and Discussion

Characterization of the Cytoplasmic and Outer Membrane Fractions. For membranes prepared from cells grown with palmitic-*13,13-d*$_2$ acid, the succinate dehydrogenase activity of the cytoplasmic membrane was 25 times that of the outer membrane and the KDO content of the outer membrane was 4 times that of the cytoplasmic membrane. The fatty acid content (Table I) of the membrane lipids of cells grown with palmitic-*13,13-d*$_2$ acid was almost identical with that of cells grown with oleic acid and palmitic-d_{31} acid (Davis et al., 1979). The palmitic-*13,13-d*$_2$ acid was indistinguishable from the completely protiated acid by GLC of the methyl esters. It was shown previously that >98% of the incorporated palmitic acid was in the phospholipids (Davis et al., 1979).

^{2}H NMR Spectra of Outer[4] and Cytoplasmic Membranes. Spectra of *E. coli* outer and cytoplasmic membrane preparations from cells grown on palmitic-*13,13-d*$_2$ acid are shown in Figures 1a–c and 2a–c. The spectrum of the outer membrane sample at 37 °C (Figure 1a) is a classic powder pattern line shape with a peak splitting of 16.5 ± 0.5 kHz. This value is in close agreement with those obtained for the fluid phase of palmitate-*13,13-d*$_2$-enriched *A. laidlawii* membranes (Stockton et al., 1977; Smith et al., 1979) and in specifically labeled DPPC (dipalmitoylphosphatidylcholine) multilamellar dispersions (Seelig & Seelig, 1974). The spectrum of the cytoplasmic membrane at 37 °C (Figure 2a) has a substantially smaller splitting of 12.0 ± 0.5 kHz. These results indicate that, at any given temperature, the fluid phase of the cytoplasmic membrane is more disordered than that of the outer membrane, in agreement with the results for palmitate-d_{31}-enriched membranes (Davis et al., 1979).

In our previous work using perdeuterated palmitic acid (Davis et al., 1979), we reported the presence of an additional feature "X" in the fluid-phase spectra of both the outer and cytoplasmic membranes. This feature is not present in the spectra of the specifically labeled membranes. Preliminary experiments on the lipids extracted from the specifically labeled membranes have shown that the outer membrane lipids, but not those of the cytoplasmic membrane, exhibit the same

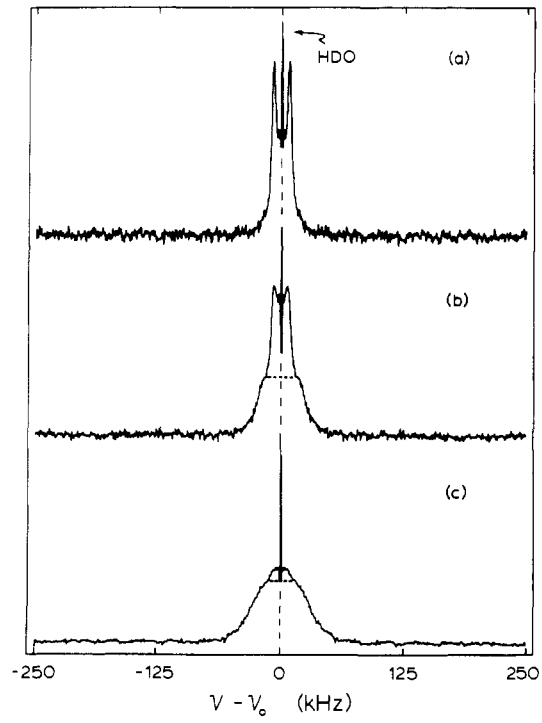

FIGURE 1: ^2H NMR spectra at 34.4 MHz of palmitate-*13,13-d*$_2$-enriched *E. coli* outer membranes. (a) $T = 37$ °C, 60000 scans; (b) $T = 25$ °C, 100000 scans; (c) $T = 5$ °C, 120000 scans. The horizontal dotted lines indicate the division of the spectra into fluid- and gel-phase components.

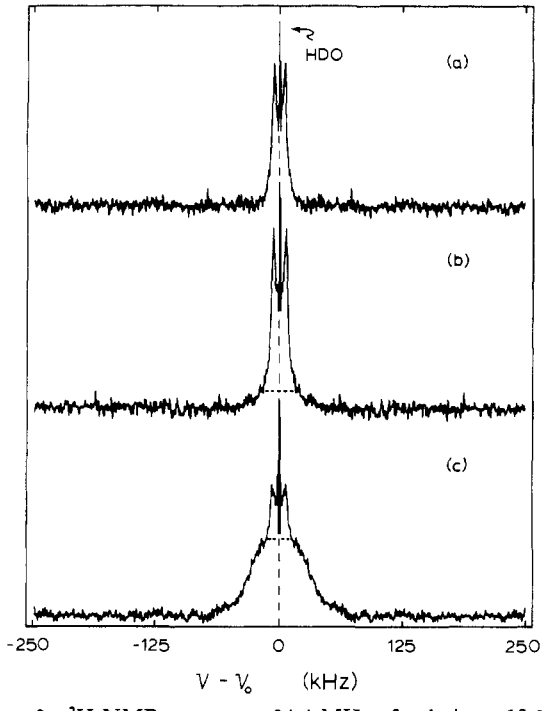

FIGURE 2: ^2H NMR spectra at 34.4 MHz of palmitate-*13,13-d*$_2$-enriched *E. coli* cytoplasmic membranes. (a) $T = 37$ °C, 60000 scans; (b) $T = 25$ °C, 60000 scans; (c) $T = 50$ °C, 150000 scans. The horizontal dotted lines indicate the division of the spectra into fluid- and gel-phase components.

behavior, above 37 °C, reported for the extracted lipids of the membranes of cells grown with perdeuterated palmitic acid (Davis et al., 1979).

At 37 °C and above, the spectra of both membrane preparations are characteristic of the fluid lamellar phase. As the temperature is lowered below 37 °C, the splitting of the fluid spectrum increases gradually as shown in Figure 3. In addition, a broad component which extends to approximately ±63 kHz increases in relative intensity as the temperature is lowered. This broad component is characteristic of the gel phase. At 25 °C, the spectrum of the outer membrane (Figure 1b) indicates that ~65% of the membrane phospholipids are in the gel phase, whereas the cytoplasmic membrane spectrum (Figure 2b) shows that only 25% of the phospholipids of that membrane are in the gel phase.

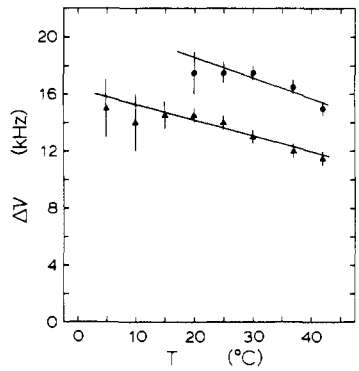

FIGURE 3: Temperature dependence of the separation between the peaks of the fluid-phase component of the spectra. The circles are for the outer membranes, and the triangles are for the cytoplasmic membranes.

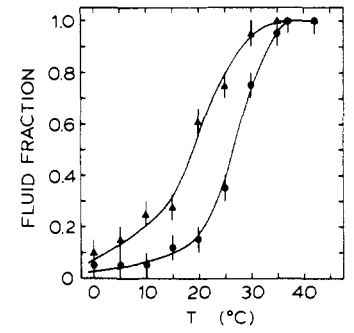

FIGURE 4: Variation with temperature of the fluid-phase fraction of the membrane lipids. Error bars are ±0.05. The circles are for the outer membranes, and the triangles are for the cytoplasmic membranes.

As the temperature is lowered further, the fraction of fluid phase decreases for both samples until less than 10% of either sample remains in the fluid phase at 0 °C. A plot of the fraction of the phospholipid molecules in the fluid phase as a function of temperature is given in Figure 4 for the two samples. At temperatures below 37 °C, the fraction of the cytoplasmic membrane in the fluid phase is larger than that of the outer membrane, in agreement with previously reported results (Davis et al., 1979).

At 5 °C and below, the spectrum of the outer membrane sample (Figure 1c) is similar in width and shape to that observed for multilamellar dispersions of specifically labeled DPPC in the gel phase at 30 °C (J. H. Davis, unpublished experiments). A similar spectrum is observed at 5 °C for the cytoplasmic membrane sample (Figure 2c), except that it has a sizable fluid-phase spectral component. It has been shown previously that the orientational order in the fluid phase of model membranes closely resembles that in the fluid phase of biological membranes (Stockton et al., 1977; Seelig & Browning, 1978). The similarity of the gel-phase spectra of the bacterial membranes to the gel-phase spectrum of DPPC suggests that model membrane systems will provide a useful basis for understanding the physical properties of the biological membrane's gel phase.

In summary, the ^2H NMR spectra presented here permit quantitative measurement of orientational order at a specific

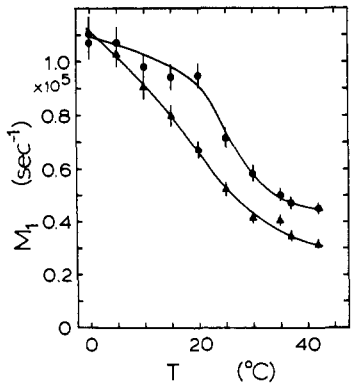

FIGURE 5: Temperature dependence of the first moment of the ^{2}H NMR spectra. The error bars are ±5%. The circles are for the outer membranes, and the triangles are for the cytoplasmic membranes.

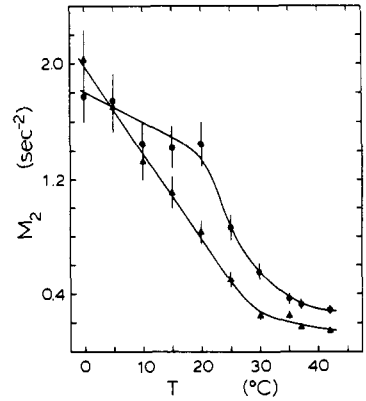

FIGURE 6: Temperature dependence of the second moment of the ^{2}H NMR spectra. The error bars are ±10%. The circles are for the outer membranes, and the triangles are for the cytoplasmic membranes.

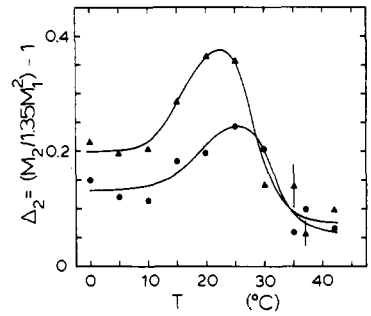

FIGURE 7: Temperature dependence of the fractional mean squared width of the distribution of quadrupolar splittings. The circles are for the outer membranes, and the triangles are for the cytoplasmic membranes.

hydrocarbon chain position. Experiments on membranes of cells grown with palmitic-*16,16,16-d*$_3$ acid gave results similar to those described above and by Kang et al. (1979b). The results reported here are consistent with those obtained for membranes prepared from cells supplemented with palmitic-d_{31} acid (Davis et al., 1979). In particular, at any given temperature within the transition region, the fraction of phospholipid molecules in the fluid phase is greater for the cytoplasmic membrane than for the outer membrane.

The fluidity of the outer and cytoplasmic membranes is certainly influenced by the acyl chain composition of the two membranes. Preliminary studies on bilayers formed from the extracted lipids of the two membranes indicate a correlation between their fluidity and that of the original membranes. The effect of the different protein compositions of the two membranes on fluidity has not been studied, but recent ^{2}H NMR studies (Seelig & Seelig, 1978; H. U. Gally, G. Pluschke, P. Overath, and J. Seelig, unpublished experiments; Kang et al., 1979a; Oldfield et al., 1978; F. W. Dahlquist, M. Paddy, J. H. Davis, and M. Bloom, unpublished experiments) suggest that a high concentration of certain proteins in a lipid bilayer reduces the orientational order of the lipids. If this is a general effect, then the higher degree of order of the outer membrane is probably not due to that membrane's altered protein concentration. One component which is found predominantly in the outer membrane is LPS. The fatty acid chains of the LPS are not contributing directly to the spectra as fatty acid supplements are not incorporated into the LPS of *E. coli* (Silbert et al., 1973b; Davis et al., 1979). The LPS does, however, form a bilayer with phospholipids in vesicles (Emmerling et al., 1977), and it is possible that the large LPS molecule may influence the order of the phospholipid acyl chains.

Analysis of the Moments of the ^{2}H NMR Spectra. For a quantitative analysis of the experimental data, the method of moments is used (see Materials and Methods). The traditional spectroscopic procedure of measuring the frequencies of sharp features of the spectrum, i.e., quadrupolar splittings, is difficult to apply when the spectra contain broad overlapping components. Even for simple spectra serious systematic errors can result if one only measures the separations of intensity maxima. The asymmetry of the quadrupolar powder line shape in the immediate vicinity of the peaks results in a decrease in the separation of the maxima whenever there is significant broadening. In spectra such as those observed in the gel phase, where there is substantial broadening, the frequencies of maximum intensity are not simply related to the mean C–D bond orientational order parameter $S_1 = \langle S \rangle$. However, the first moment of the spectrum, M_1, does give S_1 directly when the broadening is due to the existence of a distribution of orientational order parameters.

Figure 5 shows the first moment of the spectrum of the outer and cytoplasmic membranes of cells grown in the presence of palmitic-*13,13-d*$_2$ acid. The values of the first moments at temperatures above 37 °C are slightly larger than the values expected for perfect quadrupolar powder patterns having the splittings $\Delta\nu$ given in Figure 3 (see eq 7). This discrepancy is of the order of 10% and has two main contributions. Firstly, as discussed above, the identification of the peak splittings with the true quadrupolar splittings, defined as the splitting between the edges of a perfect unbroadened quadrupolar powder pattern (Seelig, 1977), leads to a systematic underestimate due to the effect of finite intrinsic line width. This effect causes an error of less than 5% for spectra such as those in Figures 1a and 2a. Secondly, the spectra may have small gel components which make important contributions to the wings of the ^{2}H NMR lines. Since the first moments of the gel spectra are several times larger than those of the liquid-crystal spectra, a very small ($\lesssim$3%) gel contribution to the total ^{2}H NMR intensity is sufficient to account for the remainder of the 10% discrepancy. Therefore, we can conclude that, on the time scale of ^{2}H NMR (10^{-4}–10^{-5} s), there are essentially no immobilized "boundary" or "annular" phospholipids (Jost et al., 1973) in the fluid phase of *E. coli* membranes. A similar conclusion was reached in a ^{2}H NMR study of *A. laidlawii* membranes (Smith et al., 1979).

The first moments increase steadily due to the increasing importance of the gel-phase contribution to the ^{2}H NMR spectrum as the temperature is lowered through the transition region. Similar remarks can be made about the second moment which is plotted as a function of temperature in Figure 6. In Figure 7, the parameter Δ_2, which gives a measure of the fractional width of the distribution of orientational order

parameters, is plotted vs. temperature for the outer and cytoplasmic membranes. As expected (Davis, 1979; Davis et al., 1979), both plots of Δ_2 exhibit maxima at temperatures at which there are approximately equal gel and liquid-crystalline contributions to the ^2H NMR spectra (see Figure 4).

Orientational Order in the Gel Phase. Using the results shown in Figures 3–6, we can now estimate the orientational order of the *gel-phase components* of the outer and cytoplasmic membranes as given by M_1^{gel} and M_2^{gel} in eq 8. We have restricted ourselves to values of $f \leq 0.8$. For higher values of f, systematic errors arising from uncertainties in f (nominally given as ±0.05 in Figure 4) are likely to be important. M_1^{gel} and M_2^{gel} (within the uncertainty of about ±10%) (a) are independent of temperature between 0 and 30 °C for the outer membrane and between 10 and 30 °C for the cytoplasmic membrane, (b) have average values which are not measurably different for the outer and cytoplasmic membranes over these temperature ranges, and (c) increase in the cytoplasmic membrane as the temperature is decreased from 10 to 0 °C.

The average values are given by

$$\langle M_1^{gel} \rangle_{cyt} = (1.13 \pm 0.09) \times 10^5 \text{ s}^{-1}$$

$$\langle M_2^{gel} \rangle_{cyt} = (1.74 \pm 0.12) \times 10^{10} \text{ s}^{-2}$$

between 10 and 30 °C and by

$$\langle M_1^{gel} \rangle_{outer} = (1.04 \pm 0.08) \times 10^5 \text{ s}^{-1}$$

$$\langle M_2^{gel} \rangle_{outer} = (1.63 \pm 0.24) \times 10^{10} \text{ s}^{-2}$$

between 0 and 30 °C.

This temperature independence of the orientational order in the gel phase of *E. coli* membranes is different from that observed in DPPC-d_{62} (Davis, 1979), where M_1^{gel} and M_2^{gel} both increase substantially between the sharp phase transition at 37 °C and 0 °C. For example M_2^{gel} increases by more than 50% over that temperature range in DPPC-d_{62}. Furthermore, the values of $\langle M_1^{gel} \rangle$ and $\langle M_2^{gel} \rangle$ are very close to those of DPPC-d_{62} *immediately below the phase transition.* Thus, the lipid–protein interaction, the phospholipid heterogeneity, and/or the coexistence of fluid- and gel-phase regions seem to stabilize the orientational order of the acyl chains at its value just below the gel to liquid-crystalline phase transition.

It is not obvious, solely on the basis of the data presented here, that the difference in the temperature dependence of M_1^{gel} and M_2^{gel} for the two membranes, below 10 °C, is statistically significant. However, for palmitate-d_{31}-enriched *E. coli* membranes (Davis et al., 1979), the variation of M_2 with temperature for the two membranes differed in the same manner as for the specifically labeled membranes at temperatures below 10 °C. It is concluded that the mechanism for the maintenance of relatively stable gel-phase orientational order as the temperature is decreased breaks down at higher temperatures in the cytoplasmic membrane than in the outer membrane.

Concluding Remarks

The ^2H NMR results on the specifically deuterated phospholipid acyl chains in the outer and cytoplasmic membranes corroborate the conclusions drawn from our earlier study of palmitic-d_{31} acyl chains. The greater spectral resolution afforded by the use of specific deuterium labels enables us to extend the interpretation of our previous work.

One of the main conclusions of our earlier study was that "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." The quantitative results presented in this paper concern this statement, and it is now possible to enlarge the discussion considerably.

(1) Above 37 °C, less than ~3% of the membrane phospholipids are in a gel or an otherwise orientationally immobilized state.

(2) At all temperatures within the transition region, the cytoplasmic membrane has a higher proportion of fluid lipid than the outer membrane. It is clear from these measurements that the transition region of the outer membrane is displaced relative to that of the cytoplasmic membrane by about +7 °C.

(3) The orientational order of the acyl chains in the fluid phase of the cytoplasmic membrane is lower than that of the outer membrane even if the displacement by 7 °C is taken into account.

(4) The orientational order of the acyl chains in the gel-phase regions of both membranes is independent of temperature over a wide temperature range. The moments (and the orientational order) of the gel-phase components of the spectra of the two membranes (between 10 and 30 °C) are identical within experimental error, and their values are similar to those of DPPC model membranes just below the phase transition (Davis, 1979). In the model membranes the DPPC molecules undergo rapid reorientational motions about their long axes at temperatures just below the phase transition, and as the temperature is lowered further there is a gradual quenching of this reorientation. It is reasonable to postulate the existence of a mechanism in *E. coli* (e.g., a lipid–protein interaction or some aspect of the coexistence of gel and fluid phases) which facilitates these reorientational motions and which is effective to lower temperatures for the outer than for the cytoplasmic membrane. ^2H NMR measurements of the extracted lipids may determine whether this property of the membranes is a consequence of its complex lipid composition or whether other membrane components (proteins or LPS) are involved.

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Binding of Scorpion Neurotoxins to Chick Embryonic Heart Cells in Culture and Relationship to Calcium Uptake and Membrane Potential†

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ABSTRACT: Stimulation of ^{45}Ca uptake by scorpion neurotoxins in cultured chick embryonic heart cells has been shown to be directly linked to their effect on sodium channels. This property was used to compare the activity of 15 neurotoxins from five different species to their lethal effect in the mouse and immunological properties. As scorpion neurotoxins, the alkaloid neurotoxin veratridine enhanced ^{45}Ca uptake, and an apparent positive cooperativity between the two drugs was observed. ^{125}I-Labeled toxin II from the scorpion *Androctonus australis* Hector was shown to bind to chick heart cells specifically, saturably, and reversibly with high affinity (K_D = 1–3 nM in sodium-free medium) and low capacity (10–20 fmol/mg cell protein). As shown by ^{45}Ca uptake and radioactive toxin binding experiments, the affinity of scorpion neurotoxin to heart cell receptors was dependent on external K^+ concentration. Toxin binding was lowered by increasing Na^+ concentration in the medium and was abolished by veratridine in a sodium (140 mM) containing medium. As previously reported for neuroblastoma cells, all these results are in agreement with the membrane potential dependence of scorpion neurotoxin affinity for its membrane receptor.

Neurotoxins isolated from African scorpion venoms are small basic proteins (Miranda et al., 1970; Rochat et al., 1979) which modify the action potential in nerve (Romey et al., 1975) and in heart cell (Coraboeuf et al., 1975) by blocking the inactivation of the sodium channel. In cultured neuroblastoma cells, they stimulate sodium transport (Catterall, 1976), increase the duration of action potential (Bernard et al., 1977), and bind to a specific membrane receptor (Catterall et al., 1976; Couraud et al., 1978). The affinity of scorpion toxin for its receptor depends upon membrane potential (Catterall et al., 1976; Catterall, 1977).

Cultured chick embryonic heart cells provide a useful system to study electrical phenomena in cardiac muscle in a nerve-free environment. Scorpion toxins were shown to induce an increase of cell beat frequency with contracture at high concentrations (Fayet et al., 1974). They stimulate the passive Na^+ and Ca^{2+} uptake (Couraud et al., 1976) and increase the duration of action potential of chick embryonic heart cells (Bernard & Couraud, 1979).

In the present work, we have investigated the effects of several toxins from five different scorpion venoms on calcium

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