J. Biochem. 54, 127–133.
Ferguson, S. J., Lloyd, W. J., & Radda, G. K. (1975c) in *Electron Transfer Chains and Oxidative Phosphorylation* (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C., & Siliprandi, N., Eds.) pp 161–166, North-Holland Publishing Co, Amsterdam.
Ferguson, S. J., Lloyd, W. J., Radda, G. K., & Slater, E. C. (1976) *Biochim. Biophys. Acta 430,* 189–193.
Fiske, C. A., & Subbarow, Y. (1925) *J. Biol. Chem. 66,* 375–400.
Frigeri, L., Galante, Y. M., Hanstein, W. G., & Hatefi, Y. (1977) *J. Biol. Chem. 252,* 3147–3152.
Godin, D. V., & Schrier, S. L. (1970) *Biochemistry 9,* 4068–4077.
Imoto, T., Johnson, L. N., North, A. C. I., Phillips, D. C., & Rupley, J. A. (1972) *Enzymes, 3rd. Ed. 7,* 665–868.
Knowles, A. F., & Penefsky, H. S. (1972) *J. Biol. Chem. 247,* 6617–6623.
Kozlov, I. A., & Skulachev, V. P. (1977) *Biochim. Biophys. Acta 463,* 29–89.
Kurzer, F., & Douraghi-Zadeh, K. (1967) *Chem. Rev. 67,* 107–152.
Levy, H. M., Leber, P. D., & Ryan, E. M. (1963) *J. Biol. Chem. 238,* 3654–3659.
Marcus, F., Schuster, S. M., & Lardy, H. A. (1976) *J. Biol. Chem. 251,* 1775–1780.
Metelsky, S. T., & Kozlov, I. A. (1974) *Dokl. Akad Nauk SSSR 219,* 1010–1013.
Mulheirn, L. J., Beechey, R. B., & Leworthy, D. P. (1974) *J. Chem. Soc., Chem. Commun.,* 874–876.
Osselton, M. D., Baum, H., & Beechey, R. B. (1974) *Biochem. Soc. Trans. 2,* 200–202.
Pedersen, P. L. (1975) *Biochem. Biophys. Res. Commun. 64,* 610–616.
Penefsky, H. S. (1967) *J. Biol. Chem. 242,* 5789–5795.
Penefsky, H. S. (1974) *J. Biol. Chem. 249,* 3579–3585.
Penefsky, H. S. (1977) *J. Biol. Chem. 252,* 2891–2899.
Racker, E., & Horstman, L. L. (1967) *J. Biol. Chem. 242,* 2547–2551.
Riordan, J. F., McElvany, K. D., & Borders, C. L. (1977) *Science 195,* 885–886.
Roberton, A. M., Holloway, C. T., Knight, I. G., & Beechey, R. B. (1968) *Biochem. J. 108,* 445–456.
Schuster, S. M., Ebel, R. E., & Lardy, H. A. (1975) *J. Biol. Chem. 250,* 7848–7853.
Webb, J. L. (1963) in *Enzymes and Metabolic Inhibitors,* Vol. I, pp 507–508, Academic Press, New York, N.Y.
Zak, B., & Cohen, J. (1961) *Clin. Chim. Acta 6,* 665–670.

Fluorine-19 Nuclear Magnetic Resonance Studies of Lipid Phase Transitions in Model and Biological Membranes†

Martin P. N. Gent‡ and Chien Ho*

ABSTRACT: Fluorinated fatty acids of the general formula $CH_3(CH_2)_{13-m}CF_2(CH_2)_{m-2}COOH$ are informative spectroscopic probes of the gel to liquid-crystalline phase transitions in phospholipid dispersions and in biological membranes. We present theoretical considerations to suggest that the ^{19}F nuclear magnetic resonance line shapes are very different for frozen and fluid lipid regions. Our studies confirm this expectation for mixed phospholipid multilamellar dispersions containing a trace of difluoromyristate. The method correctly measures the onset and completion temperatures of the transition in the well-studied dimyristoylphosphatidylcholine–distearoylphosphatidylcholine system and also describes the motional behavior of the solid and fluid phases within the transition. Lipids extracted from *Escherichia coli* membranes show similar motional phenomena through the transition-temperature range according to ^{19}F nuclear magnetic resonance studies of difluoromyristate biosynthetically incorporated into the K1060B5 strain, an unsaturated fatty acid auxotroph. Intact cells or membrane vesicles show substantially different behavior from extracted lipids, indicating that membrane proteins significantly perturb the phase transition. Evidence presented in this paper also shows that the ^{19}F resonance from *Escherichia coli* phospholipids is sensitive to various intramembrane interactions. There is a general decrease in restriction of motion due to neutral lipids and an opposite effect due to the architecture of the native membrane. Neither effect is temperature sensitive. However, there are interactions in the intact membrane, affecting the ^{19}F resonance, that are temperature dependent both due to the phase-transition process and due to processes occurring at high temperatures.

Enzymatic processes within and transport across biological membranes show a temperature dependence that can be correlated to the fluidity of the phospholipid component of the membrane. For *Escherichia coli* cytoplasmic membranes, in particular, or the isolated lipids in aqueous dispersions, there are discrete changes in the slope of the temperature dependence of the specific heat, molar volume, or the intensity of a sharp X-ray diffraction line (Overath and Trauble, 1973; Overath et al., 1975; Linden et al., 1977; Jackson and Sturtevant, 1977). These changes occur at about the same temperature in both intact membranes and isolated lipids, implying that a change in the state of the lipids is being measured. Furthermore, there are changes in the temperature dependence of membrane-related enzyme activity, lactose transport or D-lactate dehy-

† From the Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. *Received November 30, 1977.* Supported by research grants from the National Institutes of Health (GM-18698 and RR-00292) and the National Science Foundation (PCM 76-21469). M.P.N.G. is a recipient of a National Research Service Award of the National Institutes of Health (CA-06038). This paper was presented in part at the 21st Annual Meeting of the Biophysical Society, New Orleans, La., Feb. 15–18, 1977, and at the 18th Experimental NMR Conference, Asilomar, Calif., April 11–15, 1977.

‡ Present address: Connecticut Agricultural Experiment Station, New Haven, Conn. 06504.

0006-2960/78/0417-3023$01.00/0

drogenase activity, that also occur at two characteristic temperatures identical to those seen in the extracted lipids (Linden et al., 1973; Morrisett et al., 1975; Thilo et al., 1977). This phenomenon strongly suggests that the motional state of the phospholipid component is a determining factor in the biochemistry of natural membranes. Such a qualitative relationship warrants careful study in the hope that the nature of the important lipid–protein interactions can be understood.

One general method for such studies involves spectroscopic probes dissolved in the hydrophobic part of the lipid bilayer to indirectly measure the physical state of the phospholipids. Such probes include nitroxide-labeled stearic acid, 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo),[1] *N*-phenyl-1-naphthylamine (NPN), and diphenylhexatriene (DPH) (Linden et al., 1973; Trauble and Overath, 1973; Morrisett et al., 1975). Anomalies in the temperature dependence of each of these probes occur during the lipid phase transition, and the information derived is similar to that for direct physical measurements. The benefits of the spectroscopic probes are that the measurements can be done easily and quickly in dilute solution under conditions that correspond to the growth of respiring cells.

One disadvantage of all the techniques mentioned so far is that they do not see solely the effects of the phospholipid. In fact, the amount of change in any macroscopic physical parameter, measured through the transition, due only to the lipid, is difficult (if not impossible) to quantitate. The ambiguity arises because changes in the physical state of the lipid will surely modulate lipid–protein interactions. The latter will register in any physical measurement of a biological membrane. The ambiguity mentioned above extends to spectroscopic probes involving nitroxide radicals or fluorescence reporter groups. These probes are added after the formation of the cell membrane and are intended to probe the interior of the bilayer. Since the probes are not phospholipids, the measurement can only report indirectly on the lipid motion. The degree to which the method sees only the phospholipids, rather than being influenced directly by proteins and/or other membrane components, cannot be determined because of the uncertainty in the location of the probe.

Another problem in using probes such as those mentioned above arises in the interpretation of the spectra in terms of the motion or physical state of the membrane phospholipids. Typically, the spectra are difficult or impossible to explain from the point of view of a rigorous theoretical interpretation. It is often simpler to draw empirical relationships, especially between the behavior of model membrane systems and biological membranes. Thus, minor but presumably significant differences in spectral properties of two membrane systems cannot be explained in a concise way and it is difficult to determine whether they correspond to important protein–lipid interactions. In most cases the use of spectroscopic probes has been limited to drawing qualitative conclusions about the state of the membrane, for instance, describing the temperatures of the onset and completion of the phase transition.

Techniques that can be more directly related to the motion of the membrane phospholipid acyl chains overcome the ambiguities mentioned above. Nuclear magnetic resonance (NMR) spectroscopy of phospholipid nuclei is one such method where a rigorous and quantitative theoretical relationship can be drawn between the observed spectra and the phospholipid motions. The relationship has been most thoroughly described for ^2H, ^{13}C, and ^1H nuclei (Seelig and Niederberger, 1974; Gent and Prestegard, 1977; Petersen and Chan, 1977). As we have discussed previously (Gent et al., 1978), there are certain experimental advantages in studying the fluorine-19 resonance of selectively labeled fluorinated phospholipids, especially in systems such as cell membranes which have a complex chemical composition. In the present paper we derive the relationship that allows the motions of the phospholipids to be precisely described in terms of the observed ^{19}F NMR spectra. We show both theoretically and experimentally that there are drastic changes in the ^{19}F spectra due to the gel to liquid-crystalline phase transition. This phenomenon can be exploited to give an accurate picture of the degree of motion and relative amounts of both solid and fluid phases throughout the phase-transition process.

To test the ^{19}F NMR method, we examine the phase transitions in multilamellar dispersions, model membrane systems consisting of one or two phospholipid components into which a small amount of difluoromyristic acid has been added. The multilayer system was chosen for study because the NMR line widths more closely approximate those in biological membranes than do the widths seen in sonicated single-walled vesicles. Small vesicles also show a strong temperature dependence of the line width and intensity that has no simple relation to the phase transition (Sheard, 1969; Horowitz et al., 1972).

The ^{19}F NMR results can be compared with those of numerous other techniques used to study multilamellar dispersions (Chapman, 1975), especially with regard to the temperature of onset and completion of the phase transition. The low concentration of the probe molecule (1%, w/w, with respect to the phospholipid) is not expected to perturb the phase-transition properties of the lipids, although high concentrations of fatty acids certainly do have a large effect (Kantor et al., 1977; J. M. Sturtevant, personal communication). In two-component systems, ^{19}F NMR gives additional information about the motional state of the lipids at temperatures within the transition range. In particular, it can be shown that discrete fluid and solid regions are present during the transition, a statement that has been postulated by other authors (Shimshick and McConnell, 1973; Lee, 1975; Mabrey and Sturtevant, 1976). Furthermore, we observe limited motions of the phospholipids at temperatures just below the phase transition. This is more evident when measured by spectroscopic probe techniques (Shimshick and McConnell, 1973; Lee, 1975; Tsong et al., 1977). They appear to be sensitive to defects in the two-dimensional crystal lattice. The present results show that the transition goes through a premelting process before truly fluid regions of lipid appear. Early studies probably have not mentioned this phenomenon because it is not as obvious as in alkanes or polyethylenes which undergo an otherwise similar transition (Templin, 1956; Mandelkern, 1964).

The phase-transition process in the intact *E. coli* membrane has also been studied by ^{19}F NMR. The ability of the ^{19}F NMR method to accurately measure the amount of fluid lipid, and its motional properties, in the presence of large amounts of frozen lipid and a high concentration of membrane protein, allows this complex process to be unravelled. It is apparent that the presence of protein greatly affects the behavior of the phospholipids. In agreement with other methods (Trauble and

[1] Abbreviations used: CSA, chemical-shift anisotropy; DPH, diphenylhexatriene; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; DSPC, L-α-distearoylphosphatidylcholine; DSC, differential scanning calorimetry; EPR, electron paramagnetic resonance; EYPC, egg yolk phosphatidylcholine; NMR, nuclear magnetic resonance; NPN, *N*-phenyl-1-naphthylamine; S_z, order parameter; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; T_1, spin–lattice relaxation time; ν_{DD}, resonance width due to static dipolar interactions; ν_0, resonance width in the absence of static dipolar interactions; ν_{CSA}, resonance width due to chemical-shift anisotropy.

Overath, 1973; Overath et al., 1975), we find that some lipids in the biomembrane do not freeze at temperatures substantially below the transition, presumably due to interactions with adjacent proteins. An unexpected result is that the presence of protein narrows the temperature range in which the majority of the lipid becomes fluid. This difference between lipid extracts and the intact membranes is surprising, since most techniques suggest that a broader transition occurs in the latter (Trauble and Overath, 1973; Jackson and Sturtevant, 1977). The ^{19}F resonance shows a premelting phenomenon that extends to temperatures well below the first appearance of distinct regions of fluid lipid. The premelting is much more gradual than that in extracted lipid membranes or any of the model systems studied. When the present results are compared to those of other techniques used to examine *E. coli* membranes of a different composition, there is sufficient evidence to show that the results of these techniques, taken as a whole, also sense the complex nature of the transition process.

Experimental Section

Materials

Chemicals. The difluoromyristic acids were synthesized from the corresponding keto acid methyl esters using a mild and specific technique involving MoF_6 in CH_2Cl_2 solution (Fluoreze M., PCR, Gainsville, Fla). For further details, see the following references: Mathey and Bensoam, 1971; Gent et al., 1977. The fatty acids used in the following experiments were judged to be pure by gas chromatography and NMR analysis. Dipalmitoylphosphatidylcholine (DPPC) was synthesized by Dr. Paulus A. Kroon of this laboratory and purified twice by silicic acid chromatography with a gradient elution of $CHCl_3$–CH_3OH. Dimyristoylphosphatidylcholine (DMPC) and distearoylphosphatidylcholine (DSPC) were purchased from Sigma. The DSPC was further purified by silicic acid chromatography. Egg yolk phosphatidylcholine (EYPC) was purified from fresh eggs (Singleton et al., 1965). All the above phospholipids were stored at −20 °C under nitrogen until used.

Preparation of E. coli Membranes. The unsaturated fatty acid auxotroph which is used to biosynthetically incorporate 8,8-difluoromyristate is strain K1060B5 (FabB, FadE, Thi^-, Str^r), kindly supplied by Dr. David F. Silbert (Silbert et al., 1973). This strain has recently been reclassified as L4-11 (Baldassare et al., 1976). The cells were grown at 37 °C in a medium containing 60 mM potassium phosphate buffer at pH 7.0, 0.01% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$, 1% casamino acids (Difco), 0.2% glycerol, 0.1% Brij-58 (Sigma), and 0.01% thiamine. The medium also contained 0.01% oleate (Nu-Check Prep), except during growth on difluoromyristate. Growth was followed by measurement of optical density at 600 nm on a Bausch and Lomb "Spectronic 20" spectrophotometer. Cells were grown to an optical density of 1.0 in oleate-containing medium and then harvested at 4 °C by centrifugation at 3000·*g*. They were resuspended in twice as much medium containing 25 μg/mL 8,8-difluoromyristate but no oleate. The cells were grown for one generation, 1 h, at 37 °C, and then harvested at 4 °C by centrifugation at 3000*g*.

The cells were immediately subject to the procedure of Kaback (1971) for the preparation of membrane vesicles. The spheroplast formation step was modified in that 30% sucrose was used in conjunction with only 50 μg/mL lysozyme. The procedure yielded uniformly small particles (vesicles) as seen by phase-contrast microscopy. These were stored at 25 mg/mL protein in D_2O buffer on ice for less than 4 days or in liquid nitrogen if kept longer than a week.

In order to examine the isolated *E. coli* lipid, vesicles or whole cells were subject to a Bligh and Dyer (1959) extraction procedure. The resulting chloroform solution was washed twice with 5% $KHCO_3$ and then dried in vacuo for longer than 4 h. Purified phospholipids were separated from the neutral lipids by an acetone-precipitation step. Twenty-five milligrams of neutral lipids was dissolved in 0.5 mL of petroleum ether. Cold acetone was added dropwise until a petroleum ether–acetone ratio of 1:9 was reached. The solution was kept at 4 °C until the solids collected at the bottom of the tube (~1 h). The acetone was decanted and the procedure repeated. The solids were dried in vacuo overnight.

Fatty acid analysis of the phospholipids by gas chromatography followed the procedure of Silbert et al. (1973). The amount of free fatty acid in the membrane vesicles was undetectable and must be less than 5% of the fatty acid acylated to the phospholipids. Protein analysis was accomplished by a modified Lowry procedure using bovine serum albumin as a standard (Lowry et al., 1951).

Samples for NMR Studies. All NMR samples were dispersed in a buffer containing 0.05 M potassium phosphate at pH 6.6, 0.01 M $MgSO_4$, and 50 μg/mL chloramphenicol. Various ratios of H_2O to D_2O were used in preparing the samples.

Vesicles or whole cells were suspended at 25 mg of protein/mL of buffer. One milliliter of sample was contained in a flat-bottomed 10-mm NMR sample tube. Extracted lipids and phospholipids were suspended at ~25 mg/mL in D_2O buffer using the same volume of sample as above. The total extracted lipids could not be dispersed by vortexing, so they were sonicated at >30 °C for ~1 min to yield a uniform white opaque dispersion. This was freeze–thawed twice to destroy any small particles resulting from sonication. The majority of the lipids then aggregated on the side of the NMR tube, leaving a faintly cloudy solution. The phospholipids when dispersed in buffer by vortexing eventually formed aggregates large enough to partially settle to the bottom of the NMR tube.

Model membranes consisting of multilayer dispersions of pure phospholipids were prepared as follows. Phospholipid (300 mg), either pure dipalmitoylphosphatidylcholine or mixtures of the various pure phospholipids, and difluoromyristate (3.0 mg) were dissolved in $CHCl_3$ and dried under a stream of nitrogen and then overnight in vacuo. The lipids were then dispersed in 0.7 mL of D_2O buffer by vortexing at temperatures greater than 40 °C. The samples were degassed and capped under nitrogen.

Methods

^{19}F NMR spectra at 84.7 MHz were taken on a Bruker HFX-90 spectrometer modified for pulse Fourier transform and equipped with a ^2H lock. The temperature of the probe was maintained at the specified temperature to within ±1 °C. A 10-mm sample tube was subject to a 90° pulse of 16 μs, and, after a dead time of 20 μs from the end of the pulse, 4K data points were accumulated at a 100-kHz rate in the quadrature-phase detection mode. The quadrature-phase accessory (Model TT-1025) was purchased from Nicolet Technology Corp. The band width of the spectrometer is approximately ±40 kHz. A repetition rate of 0.9 s was used to allow the ^{19}F magnetization to recover between pulses. Typically 10K scans were accumulated in 2.5 h. The signal was enhanced with an exponential multiplication of 50 Hz and Fourier transformed to 2K points in the real domain. The figures in this paper show a 20-kHz segment of the 50-kHz sweep width centered 10 kHz from the transmitter frequency. A spectrum of a lipid sample containing no fluorine was subtracted from each spectrum to

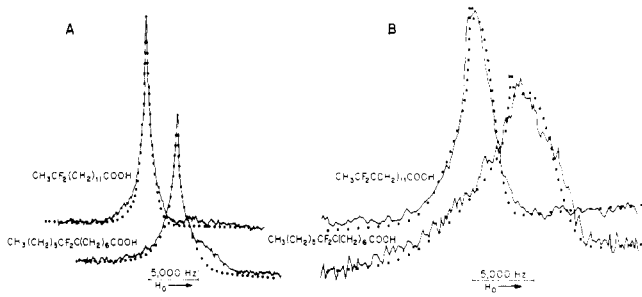

FIGURE 1: ^{19}F NMR spectra of fluorinated myristic acid in egg yolk phosphatidylcholine multilayers: (A) 84.7-MHz ^{19}F NMR spectra at 37 °C; (B) 235.2-MHz ^{19}F NMR spectra at 35 °C. (-) Experimental spectrum; (·) computer simulated spectrum (for details, see the text).

reduce an undulating baseline due to "pulse breakthrough". The spectra were corrected for the frequency dependence of the phase using a standard with several sharp lines distributed across the measured frequency range.

^{19}F NMR spectra at 235.2 MHz were taken on the MPC-HF 250-MHz NMR correlation spectrometer (Dadok and Sprecher, 1974). A modulation frequency of 50 kHz was used to sweep a 30-kHz sweep width at 0.5 s/scan. Typically, 10 000 scans were required to see spectra from membrane vesicles in a 5-mm sample tube. For weak signals, a baseline correction had to be applied. No frequency-dependent phase correction was necessary. The temperature of the probe was 35 °C.

Theoretical Section

The ^{19}F resonance derived from a difluoromethylene-labeled fatty acid in a phospholipid bilayer membrane can be explained in terms of fundamental principles of magnetic resonance as applied to the restricted motion of the fluorine nucleus. There is substantial agreement on the theoretical basis of the spectra seen for various nuclei attached to phospholipids. For instance, ^{1}H or ^{13}C NMR spectra can be explained purely in terms of intramolecular dipolar interactions that are not averaged out on the NMR time scale of $\sim 10^{-5}$ s (Gent and Prestegard, 1977; Petersen and Chan, 1977; Bloom et al., 1977). From the measurement of the width of the resonance, an order parameter (S_z) can be defined for the dipolar interaction, which can be used to describe the motion of the phospholipids.

The nonaveraged chemical-shift anisotropy (CSA) of the ^{31}P nucleus or the quadrupole splitting of ^{2}H nuclei defines an order parameter for the motion of the region of the phospholipid to which these nuclei are attached (Seelig and Niederberger, 1974; Niederberger and Seelig, 1976; Kohler and Klein, 1977). In this section, we shall show that the ^{19}F resonance can be understood in a similar manner. The only significant complexity is that both dipolar and chemical-shift anisotropy interactions are equally important at the resonance frequency of our experiments, 84.7 MHz. The importance of both of these interactions is clearly displayed in the magnetic field dependence of the line shape. At 84.7 MHz the line shape is asymmetric about a sharp central spike (Figure 1A), whereas at 235.2 MHz a triangular shape similar to that for an axially symmetric chemical shift anisotropy tensor is seen (Figure 1B). Dipolar and CSA interactions are distinctly different and they will be discussed separately before describing their combined effects. There are other magnetic interactions to consider, such as dipole-dipole interactions between nuclei on adjacent chains, but these are averaged to zero in less than 10^{-5} s. Therefore, they have only secondary effects on the line shape which will be described later.

Incompletely averaged dipolar interactions lead to a broad resonance with a Gaussian line shape whose width depends on the orientation of the phospholipid fatty acyl chain. Except in completely frozen phospholipids, there will be some averaging of dipolar interactions due to restricted motions of the hydrocarbon chains. In particular, rotation will average to zero the projection of the dipolar interaction vector in the plane perpendicular to the chain axis, leaving the projection parallel to the axis. Thus, all dipolar interactions for a given hydrocarbon chain will be scaled according to the values of $0.5(3\cos^2\theta - 1)$, the angular dependence of the dipole-dipole interaction strength, where θ is the angle between the chain axis director and the applied magnetic field (Wennerstrom, 1973). Each chain is assumed to maintain the orientation described by θ for a time longer than 10^{-5} s, so a line width for any specific orientation can be defined by

$$\sqrt{M_2(\theta)} = \sqrt{M_2(0)}\left(\frac{3\cos^2\theta - 1}{2}\right) \quad (1)$$

where $M_2(\theta)$ corresponds to the second moment or the mean squared line width for a Gaussian line shape. The value for $M_2(0)$ can be derived a priori from a knowledge of the gyromagnetic ratios, γ, and internuclear distances, r, of the difluoromethylene group and adjacent protons (Abragam, 1961).

$$M_{2,\mathrm{FF}}(0) = \frac{3}{4}\gamma_F{}^4\hbar^2 I(I+1)\left(\frac{1\text{-fluorine}}{r_{\mathrm{FF}}{}^6}\right) = 2.25 \times 10^9\ \mathrm{rad}^2/\mathrm{s}^2 \quad (2)$$

$$M_{2,\mathrm{HF}}(0) = \frac{1}{3}\gamma_F{}^2\gamma_H{}^2\hbar^2 I(I+1)\frac{8\text{-protons}}{\langle r_{\mathrm{HF}}{}^6\rangle} = \sim 4.1 \times 10^9\ \mathrm{rad}^2/\mathrm{s}^2 \quad (3)$$

where $r_{FF} = 2.18$ Å for model compounds, and $\langle r_{HF}\rangle \approx 2.5$ Å for each of the eight protons on the four nearest methylene groups. Other symbols have their usual meanings and values.

In the liquid-crystalline phase of phospholipids there are additional molecular motions superimposed on rotation that will reduce the time-averaged value of the projection of the dipolar interaction vector along the chain axis. This reduction is described by an order parameter, S_z, varying between zero and one. In the presence of restricted molecular motions, the line width is given by:

$$\nu_{\mathrm{DD}}(\theta) = \sqrt{M_{2,\mathrm{FF}}(0) + M_{2,\mathrm{HF}}(0)} \times S_z\left(\frac{3\cos^2\theta - 1}{2}\right) \quad (4)$$

Nonaveraged CSA interactions cause a shift in frequency of the resonance that depends on the orientation of the chain axis. Although the chemical-shift tensor in difluoromyristate has not been determined, it can be approximated by that for other compounds containing a difluoromethylene group. The magnitude and direction of the CSA in the molecular framework in Teflon and silver trifluoroacetate are similar, suggesting that it is invariant for all compounds containing a sp^3-hybridized carbon atom. Using the measured traceless CSA tensor in Teflon (Mehring et al., 1971) to approximate that in difluoromyristate, three distinct elements are found: $\sigma_{11} = -81$, $\sigma_{22} = +21$, $\sigma_{33} = +59$ ppm, where σ_{33} is along the C-F bond direction and σ_{11} is perpendicular to this in the C-C-F plane. A rotation of the coordinate system by 30° around the 33 axis orients the 11 direction parallel to the hydrocarbon chain axis. This rotation is illustrated in Figure 2, in which the tetragonal difluoromethylene group is shown from several angles while superimposed within a cubic lattice.

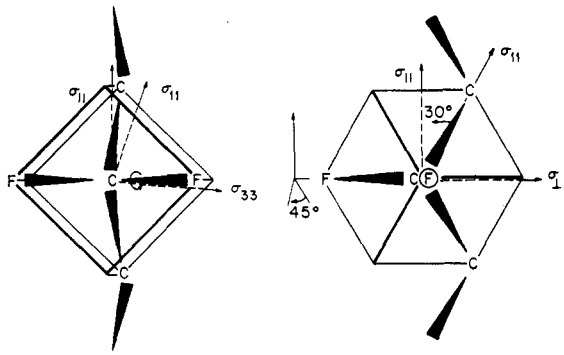

FIGURE 2: Tetragonal representation of the difluoromethylene group superimposed on a cubic lattice illustrating the coordinate transformation of the chemical-shift anisotropy tensor to the molecular long axis.

Subsequent motional averaging due to rotation about the chain axis yields a pseudoaxial symmetry of the chemical-shift tensor.

$$\sigma_{\parallel} = \sigma_z = \sigma_{11} \times \cos 30° + \sigma_{22} \times \sin 30° = -59 \text{ ppm} \quad (5)$$

$$\sigma_{\perp} = \langle \sigma_x + \sigma_y \rangle = 0.5\sigma 33 + 0.5(-\sigma_{11} \times \sin 30° + \sigma_{22} \times \cos 30°) = +59 \text{ ppm} \quad (6)$$

Fluid phospholipids will have a maximum CSA of 118 ppm.

For a specific orientation of the fatty acyl chain, θ, and the Larmor frequency, ν_0, the resonance frequency will be

$$\nu_{CSA}(\theta) = \nu_0 + \frac{2}{3}\nu_0(\sigma_{\parallel} - \sigma_{\perp})\left(\frac{3\cos^2\theta - 1}{2}\right) \quad (7)$$

Thus, at a given orientation the shift in frequency due to CSA interactions will be proportional to the broadening caused by dipolar interactions. The quantity $(\sigma_{\parallel} - \sigma_{\perp})$ corresponds to the projection of the chemical-shift tensor along the axis of the chain. For this reason it will be reduced by molecular motions, other than simple chain rotation, in the same way that dipolar interactions are reduced.

It is apparent that if rotation of the fatty acid around the chain axis is assumed further molecular motions will not change the ratio of dipole–dipole to CSA interaction strength. Therefore, once this ratio is determined at a given Larmor frequency, it is no longer a variable. The numbers used above predict a ratio of 1:1 at 84.7 MHz, while the ratio found by spectral simulation is 0.8:1 at this frequency. In order to account for this discrepancy when deriving order parameters from the observed line shapes, the following values for the static interaction strengths have been used: $(\sigma_{\parallel} - \sigma_{\perp}) = 110$ ppm; $\sqrt{M_{2,DD}} = 15$ kHz.

Certain assumptions implicit in the theory stated above may lead to systematic errors in the order parameter, S_z, derived from the line shape. They also lead to the discrepancies between theory and experiment mentioned above. These assumptions are: the "order parameter" for the adjacent methylene ^{1}H–^{19}F dipolar interactions is the same as the "order parameter" for motion of the difluoromethylene group itself; the coupling between CSA and dipolar interactions (van Willigen et al., 1977) results in a Gaussian line shape, after rotational averaging; and the traceless CSA tensor in difluoromyristate is the same as in Teflon.

Under strong proton decoupling (such that ^{1}H–^{19}F dipolar interactions are averaged on a time scale of $\sim 10^{-6}$ s), the line shape due only to the difluoromethylene magnetic interactions would be seen (Urbina and Waugh, 1974). This would sharpen the resonance and allow a more exact analysis of the order parameter. However, such decoupling is difficult because a radiofrequency field of ~10 G is needed for full decoupling. The 6% difference between ^{1}H and ^{19}F resonance frequencies causes the decoupling field to overwhelm the detection of the ^{19}F signal. We present a simpler technique because it is less difficult and requires less sophisticated hardware.

We have resorted to simplifications to extract some information from the resonance. The line shape does fit the resonance reasonably well so the fundamental assumption of pseudoaxial symmetry for the magnetic interactions is correct. A systematic error in the relative magnitude of the various contributions (CSA, F–F dipolar, and H–F dipolar) can occur. This is confirmed by the fact that theoretically derived values are ~15% different than the values needed to accurately fit the observed resonance. Thus, it would be reasonable to suggest an accuracy of 20% in the absolute value of the order parameter. The precision of the method, comparing order parameters derived from ^{19}F NMR experiments, is better because the errors resulting from poor values for these magnetic interaction components would scale as S_z.

Given values for ν_{CSA} and ν_{DD}, a line shape can be calculated by integrating over all possible values of θ (see for instance, Niederberger and Seelig, 1976). The shape predicted by this procedure actually corresponds to an envelope or sum of individual resonances, each corresponding to a particular angle for θ. If ν_{CSA} is less than ν_{DD}, this line shape has an infinitely sharp central spike at the point where $[(3\cos^2\theta - 1)/2] = 0$. Experimentally, the ultimate narrowing of the central spike is equivalent to a width of about 50 Hz at 84.7 MHz. This width, ν_0, is due to dipolar interactions that are averaged to zero in less than 10^{-5} s in fluid lipid regions. Two possible sources of this broadening are transverse relaxation due to nuclei on phospholipids diffusing past one another, and relaxation due to intramolecular interactions that fluctuate quickly due to fatty acid chain motions. These phenomena give rise to a line broadening that is independent of the fixed orientation of the fatty acid axis and thus do not scale as $(3\cos^2\theta - 1)$, like the orientation-dependent phenomena described previously.

The theory can be used to predict the line shape of the ^{19}F resonance of a difluoromethylene-labeled fatty acid in a lipid bilayer at any applied magnetic field. The resonance will depend upon ν_{CSA}, ν_{DD}, and ν_0 where ν_{CSA}/ν_{DD} is fixed at a given Larmor frequency. The Fortran computer program used to calculate and display the line shape is found in the supplementary material to this paper. Since ν_{CSA} increases proportional to the applied magnetic field, the theory is tested at two magnetic field strengths. Spectral simulation of the resonance of difluoromyristate incorporated into egg yolk PC membrane at 84.7 and 235.2 MHz is shown in Figure 1. An excellent fit is observed at both field strengths without changing any parameter except the Larmor frequency. This result strongly supports the validity of the theory described above.

At a resonance frequency of 84.7 MHz, ν_0, describing the ultimate narrowing of the central spike, is a parameter for which the line shape is quite sensitive. Thus, the resonance provides information on dynamic processes independent of the order parameter governing ν_{DD}. However, because both parameters are important in determining a line shape, a precise determination of both is difficult unless a noise-free, undistorted signal can be seen. For this reason we cite a confidence interval of ±10% for ν_{DD}, ν_0, and S_z from measurements taken at 84.7 MHz.

At 235.2 MHz, ν_0 has very little effect on the line shape. Furthermore, the limits of the resonance are clearly defined. For these reasons, ν_{DD} can be accurately determined. The precision of the order parameter at this higher frequency should be better than 2%. The separation of the components

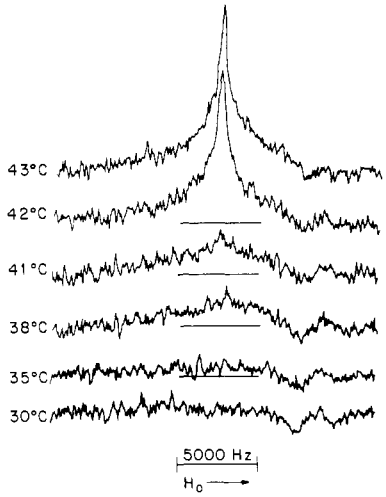

FIGURE 3: 84.7-MHz ^{19}F NMR spectrum of DPPC multilayers as a function of temperature. Experimental conditions were: 0.2 wt % 8,8-difluoromyristate and 25 wt % dipalmitoylphosphatidylcholine in D_2O buffer; 10 000 scans per spectrum.

of the CSA tensor at higher magnetic fields will finally resolve the time-averaged components from the dipolar interactions so that the frequency shift can be measured. Thus, ideally, measurements of the ^{19}F resonance shape in bilayer membranes should be taken under identical conditions at two different field strengths in order to extract the greatest amount of information.[2]

Due to the complex interdependence of ν_{DD} and ν_0 at 84.7 MHz, there is no simple measurement of the resonance that is linearly related to the parameters describing the line shape. Thus, the resonance width at half-height is not proportional to ν_{DD}, and the height is not proportional to the intensity. This behavior means that spectral simulation is essential in order to interpret the observed ^{19}F resonance.

^{19}F NMR line-shape studies of difluoromethylene fatty acids in bilayer membranes should be a direct and accurate measurement of the motional state of the phospholipid during the phase transition. Very different resonances are postulated for phospholipids in the liquid-crystalline and gel states. The former is characterized by a narrow resonance whose shape parameters define the restriction of motion, S_z, and give information on the dynamic aspects of motion through ν_0. In contrast, the resonance from the gelled lipids is broad, greater than 7-kHz wide, and does not have a sharp central spike. This is easily understood theoretically because the molecular motions that cause narrowing of the resonance are almost completely eliminated in the gel state. Thus, both ν_{DD} and ν_0 become larger and approach the values in a solid. The ν_0 parameter probably has a contribution from nonaveraged intermolecular interactions, due to the cessation of diffusional motions, that increase the width of the central spike to 1–2 kHz. Because of the very different line shapes from the liquid crystalline and gel states of the phospholipids, the relative intensities and the line-shape parameters of each can be accurately measured, even in situations where both phases exist simultaneously. In particular, the intensity of the resonance due to fluid lipid and its order parameter can be easily measured for any temperature within a phase transition.

[2] We were unable to take advantage of the comparison of the ^{19}F resonance at the two frequencies 84.7 and 235.2 MHz. This is because a variable temperature accessory is not available on the higher frequency spectrometer.

The intensity of the ^{19}F resonance is affected both by the spin–lattice relaxation time (T_1) and nuclear Overhauser effects, phenomena that have been described in a previous publication (Gent et al., 1976). Due to the long recovery time used in the present experiments, 0.9 s, the short T_1 of fluid lipids, 0.3 s at 50 °C, and the lack of proton radiofrequency irradiation, the intensity of the fluid lipid will correspond to the equilibrium fluorine magnetization. However, in completely frozen or immobile lipids, T_1 could become long enough to cause saturation under the experimental conditions used. This will result in the complete disappearance of the ^{19}F NMR signal regardless of the spectrometer bandwidth. It should be noted that the broad resonance that is often stated as due to frozen lipid, actually corresponds to lipids which have sufficient motion for recovery of the fluorine magnetization, i.e., $T_1 \leq 1$ s. Without giving the details, it can be shown that in order to have such a broad resonance and effective spin–lattice relaxation, the phospholipids involved must either undergo extremely restricted, but relatively rapid motion (faster than 10^{-8} s), or there must be exchange of lipids between frozen and fluid domains with an average exchange rate of $\ll 1$ s. The present experiments cannot distinguish between these two possibilities.

Results

^{19}F NMR Studies of Model Membrane Systems

Multilamellar Dispersions of DPPC. L-α-Dipalmitoylphosphatidylcholine (DPPC) is a single isomer of defined composition. Since it is a pure compound, it is expected to undergo an abrupt phase transition. The model membrane consisting of DPPC dispersed in excess water, in a multilamellar array, does indeed show an abrupt transition from a gel to a liquid-crystalline state at 41.4 °C that is only 0.2 to 0.4 °C wide (Mabrey and Sturtevant, 1976; Nagle and Wilkinson, 1978). Most physical techniques also show a pretransition at or about 35 °C. Gradual premelting before the main transition is uncertain. Some techniques show a large change, while others show little or no change with temperature within 5 °C of the transition.

An examination of the temperature dependence of the ^{19}F resonance of 8,8-difluoromyristate incorporated into a DPPC multilamellar dispersion reveals the dramatic changes induced by the gel to liquid-crystalline phase transition. Figure 3 shows the ^{19}F resonance in the range from 35 to 60 °C. Below 35 °C no resonance is seen, given the limitation of the NMR spectrometer and repetition rate for data accumulation. Between 35 and 41 °C there is a gradual appearance or increase in the intensity of a very broad resonance, estimated to be 5–10 kHz wide. At 41 °C the integrated intensity of the broad resonance has increased to that of the sharp resonance seen at high temperatures. Between 41 and 42 °C a relatively sharp resonance suddenly appears, that is similar in every way to the one seen in fluid EYPC membranes (Figure 1). Above 42 °C the resonance narrows slightly with temperature and the height increases in a gradual manner.

If it is assumed that the fluorinated fatty acid probe exactly follows the behavior of the surrounding phospholipids, the NMR data are easy to explain within the framework of the ^{19}F resonance relaxation theory proposed. Below 35 °C, there is no motion of the probe so the dipolar line widths will be at a maximum of 15 kHz. Also, the width of the central spike would be very broad, 1–2 kHz. Although such a resonance is theoretically detectable on our NMR spectrometer, it is probably saturated due to inefficient spin–lattice relaxation. Above 42

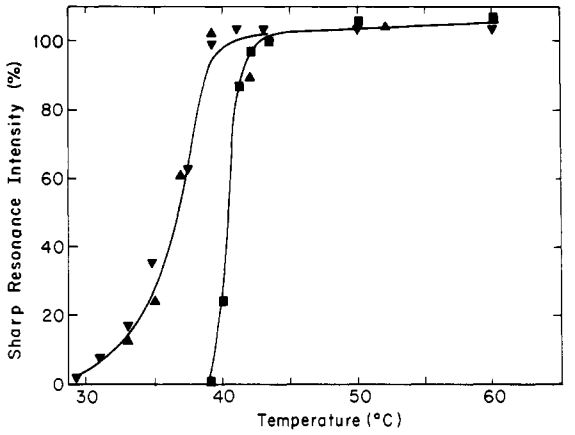

FIGURE 4: The intensity of the narrow 84.7-MHz ^{19}F resonance of difluoromyristate in model membranes: (■) 0.2 wt % 8,8-difluoromyristate in 25 wt % dipalmitoylphosphatidylcholine; (▲) 0.2% 8,8-difluoromyristate in 20 wt % dipalmitoylphosphatidylcholine plus 5 wt % egg yolk phosphatidylcholine; (▼) 0.2% 13,13-difluoromyristate in 20 wt % dipalmitoylphosphatidylcholine plus 5 wt % egg yolk phosphatidylcholine.

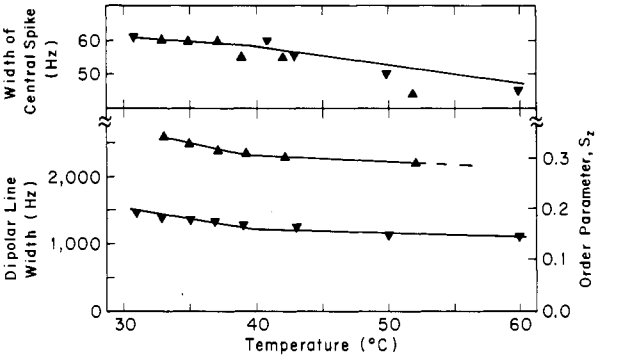

FIGURE 5: The width parameters of the narrow ^{19}F resonance of difluoromyristate in DPPC–EYPC mixtures as a function of temperature: (▲) 0.2% 8,8-difluoromyristate in 20% dipalmitoylphosphatidylcholine plus 5% egg yolk phosphatidylcholine and (▼) 0.2% 13,13-difluoromyristate in 20% dipalmitoylphosphatidylcholine plus 5% egg yolk phosphatidylcholine.

°C the probe molecule would be as free to move as the surrounding fluid phospholipids. The rotation, translation, and bond isomerization motions result in a relatively sharp resonance. At higher temperatures, these motions become faster and less restricted, leading to further narrowing of the resonance. Whatever transition occurs at 35 °C does seem to lead to the onset of some motion of the phospholipid fatty acyl chains, but it is not the rapid and continuous isomerizational motion seen in the liquid-crystalline state. The ^{19}F resonance of the probe molecule does not show a narrow component appearing at this temperature.

The ^{19}F resonance at any temperature is arbitrarily decomposed into the sum of two resonances of different widths. One is very broad and corresponds to the frozen lipid. The other, much sharper, corresponds to the fluid lipid. Figure 4 shows the temperature dependence of the intensity of the sharp part of the resonance of difluoromyristate in DPPC. As expected, there is an almost discontinuous jump from 0 to 100% intensity over a 1 °C range in temperature at 42 °C. At higher temperatures there is no further increase in intensity. The increase in height of the resonance is offset by the corresponding decrease in the width. The underlying dipolar width and static width of the sharp part of the resonance show changes with temperature above 41 °C identical to those shown in Figure 5 for a different lipid mixture. The order parameter for the difluoromethylene group changes very little in this temperature range. But there is a more significant narrowing of the sharp central spike as measured by ν_0. These effects are presumably brought about by the slight expansion of the bilayer membrane with temperature and by the increase in rate of molecular motions.

Multilamellar Dispersions of a Mixture of DPPC and EYPC. A mixture of DPPC and EYPC is the first step along the continuum from the homogeneous DPPC dispersion to the heterogeneous lipid mixtures seen in biological membranes. EYPC has the same head-group structure and configuration as DPPC. One of the fatty acid chains is usually palmitate. The other chain is often an unsaturated fatty acid, typically oleate or linoleate. A mixture of 80% DPPC and 20% EYPC thus has a fatty acid composition of about 90% palmitate and 10% various other fatty acids. The introduction of this slight heterogeneity of lipid composition, especially the unsaturated fatty acids, is expected to decrease the phase-transition temperature and to increase the temperature range between the onset and completion of the transition. Because of the very similar nature of the two phospholipids, there will be no phase separation of the two components during the transition.

These expectations are fulfilled according to the ^{19}F resonance of a fluorinated fatty acid probe (Figure 4). Visual inspection of a series of spectra of either 8,8- or 13,13-difluoromyristate incorporated to 1% (w/w) in 80% DPPC and 20% EYPC shows a narrow resonance appearing at 31 °C and growing in height continuously to 39 °C. Above 39 °C there is a much more gradual increase in height. Below 31 °C there is no detectable narrow resonance. There are no abrupt increases in height at the beginning or end of the presumed phospholipid phase transition that would indicate a partitioning of the probe into one or the other of the components of the lipid mixture. The probe is uniformly dispersed through the bilayer and reports on the motion of a random sample of the phospholipids.

The apparent width of the ^{19}F resonance does not significantly decrease through the course of the transition. The 8,8 and 13,13 isomers of difluoromyristate have very different resonance widths, indicating that they monitor different regions of the bilayer and report on the "mobility gradient" of the phospholipid fatty acyl chains (Figure 5). Nevertheless, the change in width of the narrow resonance as the model membrane goes from a completely frozen to a fluid state is small for both probes.

These results suggest that the difluoromyristate probe is found in two distinct regions in the bilayer membrane. One region is frozen while the other is as fluid as the membrane above the high-temperature end of the transition. The line-fitting procedure can be used to state this result more clearly. If spectra are synthesized using a superposition of a broad resonance corresponding to frozen lipids and a sharp resonance corresponding to fluid lipids, a very good fit is obtained to a series of spectra of difluoromyristate in 80% DPPC and 20% EYPC at various temperatures. Throughout the transition the total intensity remains constant, and the widths of the two spectral components are also constant. If it is assumed, instead, that all the lipids are undergoing the same motion at any given point within the transition, then the increase in height will result in a drastic narrowing of the resonance in order to keep the intensity constant. From this assumption a simulation of the observed spectra is approached by using a single resonance of constant intensity. This gives a very poor fit. Thus, the data are more correctly interpreted in terms of a two-domain model with very different spectra arising from each lipid domain.

The spectra of the mixture of 80% DPPC and 20% EYPC

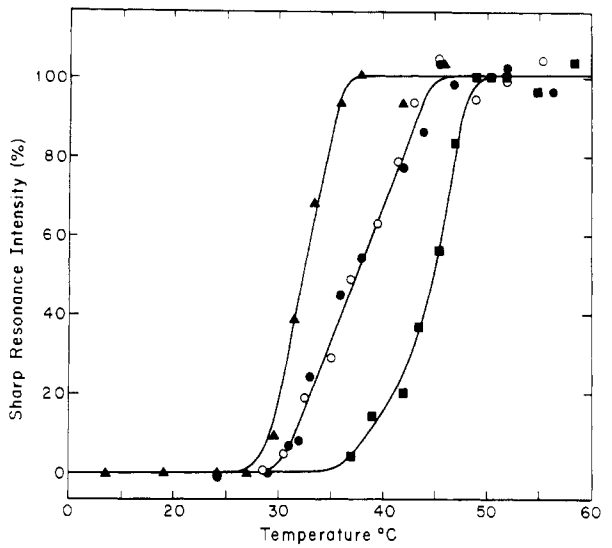

FIGURE 6: The intensity of the narrow 84.7-MHz ^{19}F resonance of difluoromyristate in DMPC-DSPC mixtures as a function of temperature: (▲) 1% (w/w) 8,8-difluoromyristate in 75:25 DMPC-DSPC; (●) 1% (w/w) 8,8-difluoromyristate in 50:50 DMPC-DSPC; (■) 1% (w/w) 8,8-difluoromyristate in 25:75 DMPC-DSPC; (○) 1% (w/w) 13,13-difluoromyristate in 50:50 DMPC-DSPC.

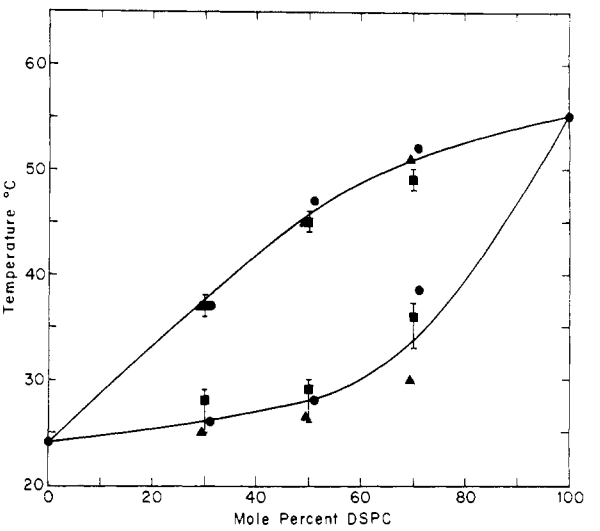

FIGURE 7: Phase diagram for DMPC-DSPC mixtures dispersed in excess water: (■) data obtained from ^{19}F NMR (this work); (●) data obtained from differential scanning calorimetry (Mabrey and Sturtevant, 1976); (▲) data collected from Tempo partition experiments (Shimshick and McConnell, 1973).

are best fit if the width parameters of the sharp resonance are allowed to change rather than being fixed throughout the temperature range. Using this procedure we have derived values for the integrated intensity of the sharp resonance, the dipolar line width (ν_{DD}), and the ultimate narrowing of the central spike (ν_0). The results are shown for both 8,8- and 13,13-difluoromyristate probes in Figures 4 and 5. The intensity vs. temperature plot of the two probes is identical (Figure 4). Thus, within the accuracy of this technique, all sections of the phospholipid molecule melt at the same time. The more loosely packed methyl ends of the phospholipid chains do not seem to undergo any motion before the carbonyl end.

Multilamellar Dispersions of a Mixture of DMPC and DSPC. Dimyristoyl- (DMPC) and distearoylphosphatidylcholine (DSPC) are both pure compounds which exhibit discrete phase transitions, in multilamellar dispersions, at 24 and 55 °C, respectively (Mabrey and Sturtevant, 1976). Mixtures of these two lipids show broad phase transitions at intermediate temperatures. If the ^{19}F resonance of the intercalated difluoromyristate correctly describes the onset and completion of the broad transitions, exclusive partitioning of the probe into either solid or fluid domains can be ruled out. This model membrane system has been studied by several techniques (Shimshick and McConnell, 1973; Mabrey and Sturtevant, 1976; Lentz et al., 1976b), so it is a stringent test of our ^{19}F NMR technique.

The temperature dependences of the spectra of 1% (w/w) difluoromyristate in mixtures of DMPC and DSPC are qualitatively the same in the DPPC-EYPC mixture as shown in Figures 4 and 6. As the temperature is increased through the range of the phase transition, a sharp resonance of approximately constant width grows in intensity. Above a certain temperature the height increases much more slowly and the integrated intensity of the resonance remains constant. The line-fitting procedure that best fits these spectra is one in which the superposition of a broad and a sharp resonance is used. The sum of the integrated intensities of these two resonances remains constant throughout the temperature range. The temperature dependence of the intensity of the sharp resonance from difluoromyristate, derived from the line-fitting procedure, is shown in Figure 6. In all three DMPC-DSPC mixtures studied there is a distinct temperature at which a sharp resonance first appears. The intensity grows gradually through the presumed range of the phase transition and then abruptly levels off. Because there is an error of ±5% in the intensity measurements, no single spectrum can be singled out as the point at which the sharp resonance intensity stops increasing. However, a simple linear extrapolation of the points at the high-temperature end of the transition accurately defines a temperature at which the transition is complete according to the difluoromyristate probe. At least for the mixture of 50:50 DMPC-DSPC, the 8,8 and 13,13 isomers of difluoromyristate show an identical narrow resonance intensity increase with temperature (Figure 6). All these characteristics of the ^{19}F resonance are most easily explained in terms of discrete regions of fluid and solid lipids within the transition-temperature range. The phase diagram described by the onset and completion temperatures of the melting of the phospholipid mixtures is given in Figure 7. Points taken from or extrapolated from the Tempo partitioning data (Shimshick and McConnell, 1973) and the differential scanning calorimetry (DSC) data (Mabrey and Sturtevant, 1976) are also superimposed. For the mixtures tested, the ^{19}F NMR method agrees remarkably well with these two methods, which measure very different properties of the phase transition. Thus, the difluoromyristate probe randomly samples all phospholipid regions in this mixed lipid model membrane system and gives a realistic picture of the phase-transition process.

For temperatures between 5 and 10 °C below the first appearance of the sharp resonance, as described in Figure 6, there is the gradual appearance of a broad ^{19}F resonance. At the temperature equivalent to the first appearance of a sharp resonance, the intensity of the broad peak is sufficient to account for all the fluorine nuclei in the samples. The phenomenon is represented by the large error bars for the solidus points on the phase diagram for the DMPC-DSPC system given in Figure 7. In this temperature region the lipids do not display a fluidlike resonance, but the ^{19}F resonance does not correspond to a complete lack of phospholipid fatty acyl chain motion either. We attribute this motion to premelting effects.

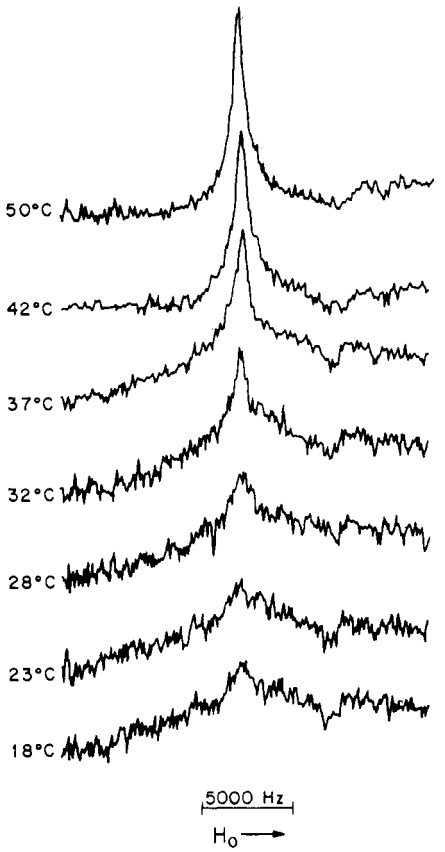

FIGURE 8 84.7-MHz ^{19}F NMR spectra of *E. coli* K1060B5 cells as a function of temperature. Cells were grown on 8,8-difluoromyristate at 37 °C. The samples for NMR studies were prepared at a concentration of 25 mg of protein/mL in D_2O buffer. Each ^{19}F NMR spectrum was accumulated over 10 000 scans.

^{19}F NMR Studies of *E. coli* Membranes

^{19}F NMR spectra can be obtained from suspensions of whole cells of *E. coli*. Figure 8 shows a series of spectra at various temperatures obtained from K1060B5 whole cells grown for one generation at 37 °C on 8,8-difluoromyristate. During the growth period the change in composition of the phospholipid fatty acids consists of a rise in the concentration of 8,8-difluoromyristate and a fall in the concentration of oleate, the usual unsaturated fatty acid supplement (Gent et al., 1978). The fatty acid composition in mol % is 12:0, 2%; 14:0, 25%; 16:0, 42%; 16:1 and cyclo 17:0, 0%; 18:1 and cyclo 19:0, 5.5%; and 8,8-difluoro 14:0, 22.5%. The growth rate at the time of harvest is similar to oleate supplemented cells and their gross morphology appears normal. The membranes are very heterogeneous in their fatty acid composition and are expected to show a typically broad lipid phase transition.

The phase transition observed by ^{19}F NMR is qualitatively different in *E. coli* membranes than in model phospholipid membranes. It is apparent in Figure 8 that at low temperatures and up to about 25 °C there is a broad but distinct ^{19}F resonance. Between 25 and 35 °C a sharper resonance appears and grows quickly in height, reminiscent of phenomena seen in pure lipid membranes. Above 35 °C the resonance continues to increase in height but rather slowly and with a concomitant narrowing of the peak. The two distinctions between the behavior in *E. coli* membranes and pure lipid membranes are that a broad resonance persists at temperatures well below the apparent phase transition and the resonance never appears to be as sharp as in model membranes until the rather high temperatures of 60 °C. The temperature dependence of the ^{19}F

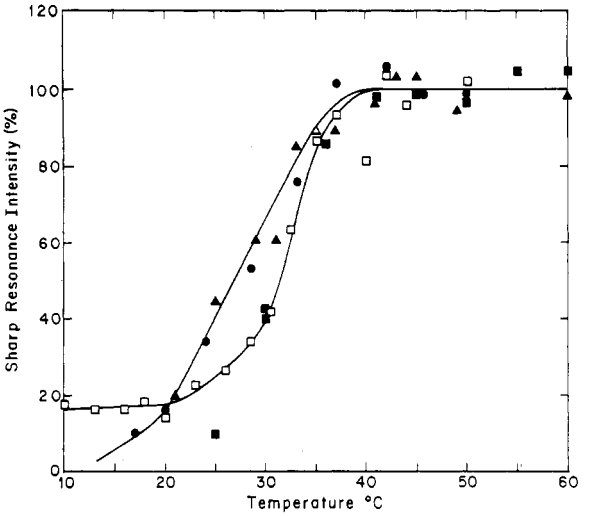

FIGURE 9: The intensity of the narrow 84.7-MHz ^{19}F resonance of 8,8-difluoromyristate in *E. coli* K1060B5 cells and extracted lipids as a function of temperature: (□) whole cells; (■) isolated *E. coli* membrane vesicles; (●) total extracted lipids; (▲) purified phospholipids.

resonance of the *E. coli* cell membranes is consistent with both fluid and solid regions of lipid existing within the range of the phase transition. If the series of spectra in Figure 8 is simulated using a superposition of broad and narrow resonances, the sum of whose intensities are constant and whose individual width parameters remain constant, a closer fit is obtained than that using a single resonance of constant intensity whose width is varied to compensate for the observed changes in the height of the experimental signal. On this basis, the ^{19}F resonance was examined as previously, using a spectral simulation consisting of two resonances. The width of the narrow resonance was varied to give the best possible fit to the experimental data. The broad resonance, nominally due to frozen lipids, is actually equivalent to lipids whose only motion is rotation about the long axis of the molecule. This spectrum is, in fact, a very good approximation to the ^{19}F resonance seen in *E. coli* membranes just below 25 °C.

The intensity of the narrow ^{19}F resonance observed in *E. coli* K1060B5 whole cell membranes analyzed as described above is shown in Figure 9. The intensity vs. temperature plot suggests that there are two distinct processes occurring during the phase transition. The rise in intensity between 10 and 25 °C is only 12% of the total intensity seen at high temperatures. The intensity rise in the following 10 °C is much more dramatic, accounting for a full 70% of the ^{19}F resonance intensity. There is no corresponding slow rise in intensity above 37 °C. Thus, the high-temperature end of the transition appears to be more abrupt than the initial melting.

Although there is an intense broad resonance superimposed on the small narrow resonance at 25 °C, this broad component gradually disappears at low temperatures. Thus, in this low-temperature region there appear to be two different processes occurring. The lipids corresponding to the broad resonance become more and more immobile, while those corresponding to the sharper resonance, about 20% of the total intensity at low temperature, eventually show no dependence of molecular motion with temperature.

Isolated cytoplasmic membranes of *E. coli* K1060B5 show the same ^{19}F resonance behavior as whole cells. The solid squares in Figure 9 correspond to spectra from cytoplasmic membrane vesicles. At all temperatures the results are the same as in intact cells. This is expected if the organization in the two membrane preparations is the same. This similarity

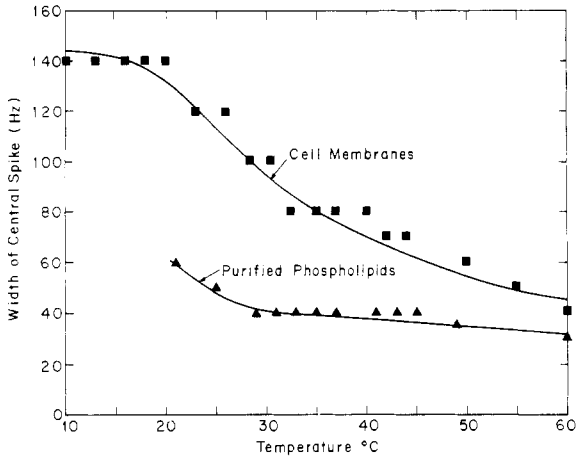

FIGURE 10: The width of the sharp central spike, ν_0, of the 84.7-MHz ^{19}F resonance of 8,8-difluoromyristate in *E. coli* K1060B5 cells and extracted lipids as a function of temperature: (■) cell membranes; (▲) purified phospholipids.

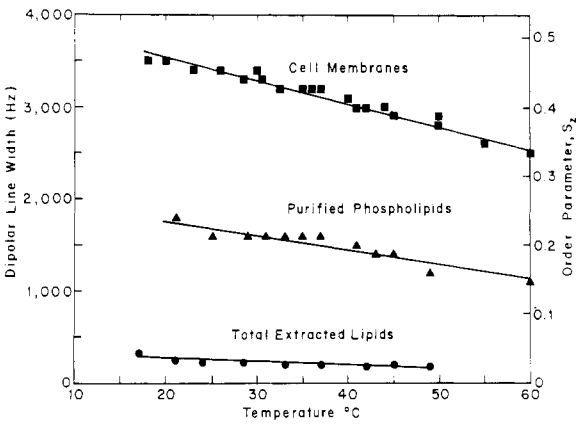

FIGURE 11: The dipolar line width, ν_{DD}, and order parameter of the 84.7-MHz ^{19}F resonance of 8,8-difluoromyristate in *E. coli* K1060B5 cell membranes and extracted lipids as a function of temperature: (■) cell membranes; (▲) purified phospholipids, (●) total extracted lipids.

emphasizes the fact that the ^{19}F resonance of the difluoromyristate is not affected by the cytoplasmic constituents of the cell in any way. The technique is only sensitive to events that change the local motion of the membrane phospholipids.

The width of the narrow resonance in *E. coli* membranes is broader than that in the pure phospholipid dispersions studied (Figure 10). The dipolar width is about 50% larger at all the temperatures studied (Figure 11). However, the relative decrease in ν_{DD} due to increasing temperature is about the same in the two lipid dispersions. In contrast, the ν_0 parameter has a very different temperature dependence in the *E. coli* membrane than in pure lipid membranes (Figure 10). The ultimate narrowing of the central spike is about 140 Hz at the lowest temperature studied and shows a decrease as the temperature is raised to 40 °C, at which point it is about 30% greater than in fluid phospholipid membranes. Above 40 °C there is a more gradual narrowing, leading to a value of ν_0 of 50 Hz at 60 °C. At this high temperature, ν_0 is approximately the same in the *E. coli* membrane and the model membranes described earlier. Up to 60 °C, ν_0 has a complicated temperature dependence in the biological membrane in contrast to an almost constant value in the model membrane.

The *E. coli* membrane lipids were extracted and redispersed in buffer solution in order to observe any differences that could

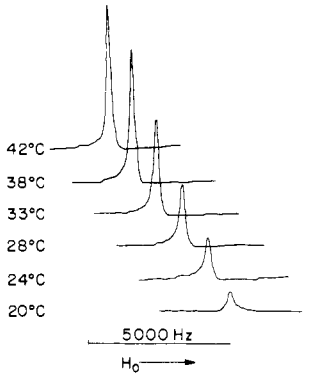

FIGURE 12: 84.7-MHz ^{19}F NMR spectra of lipids from a Bligh and Dyer extraction of *E. coli* K1060B5 cells as a function of temperature. Cells were grown on 8,8-difluoromyristate at 37 °C. The lipid concentration was 25 mg/mL in D_2O–H_2O buffer. Each spectrum was accumulated over 4000 scans.

be ascribed to protein–lipid interactions. The total lipid extract was dried in vacuo to remove all traces of organic solvent and then dispersed in buffer using a short period of sonication. The resulting solution gave a very narrow ^{19}F resonance of ~200 Hz width at half-height. Since the line width is so much narrower than in the membranes, it seemed likely that sonication created particles small enough to narrow the resonance. The effect is well documented for ^1H, ^{13}C, and ^2H NMR resonances of sonicated phospholipid dispersions (Stockton et al., 1976; Gent and Prestegard, 1977; Peterson and Chan, 1977). The *E. coli* lipid extract was freeze–thawed several times to break up vesicular structures. This procedure caused the lipids to aggregate on the walls of the sample tube, leaving the solution absolutely clear. The visual evidence suggested that no small sonicated lipid particles remained. Nevertheless, the ^{19}F resonance of the freeze–thawed sample continued to be very narrow as shown in Figure 12.

It is unlikely that the pure phospholipids of *E. coli* have such a narrow line width. These phospholipids, mostly phosphatidylethanolamine and phosphatidylglycerol, seem to behave similarly to phosphatidylcholine in model membrane systems studied by various physical techniques. In particular, the NMR line widths or electron paramagnetic resonance (EPR) order parameters are not very different in model membranes containing different types of lipids. In the present case, however, the total lipid extracts of *E. coli* membranes have a ^{19}F resonance ten times narrower than in the lipid model membranes studied. Probably some component of the lipid extract greatly disorders the phospholipids in this dispersion. To test this, the phospholipids were purified by an acetone-precipitation step, which is expected to remove all neutral lipids in the mixture, and dispersed in buffer without sonication.

Figure 13 shows that the purified phospholipids of *E. coli* have a resonance shape and width much more similar to those of both the intact *E. coli* membranes and model membranes than to those of the total lipid extracts. The line width is still not as wide as in the model membranes but this could well be due to the presence of a trace of those components that narrow the resonance so dramatically in the total lipid extracts.

The temperature dependence of the ^{19}F resonance of both extracted lipid dispersions derived from the *E. coli* membrane qualitatively shows the trends seen in the other model lipid dispersions. A narrow resonance appears that increases in height throughout the range of the phase transition without any apparent change in width. Above the transition, very little increase in height with temperature is seen. As previously discussed, this behavior indicates that within the transition

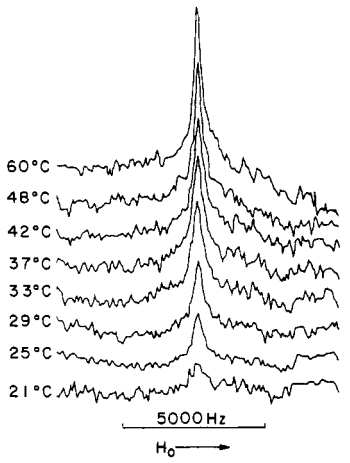

FIGURE 13: 84.7-MHz ^{19}F NMR spectra of purified phospholipids of *E. coli* K1060B5 cells as a function of temperature. Cells were grown on 8,8-difluoromyristate at 37 °C. The phospholipid concentration was 20 mg/mL in D_2O–H_2O buffer. Each spectrum was accumulated over 10 000 scans.

discrete regions of fluid and solid lipids exist. The resonance arising from each is sufficiently different for them to be resolved and analyzed using the two-component line-fitting procedure. The intensities of the narrow resonance of the two *E. coli* lipid dispersions, derived from the spectral simulation, show an identical temperature dependence (Figure 9). Despite the very different molecular motions in the fluid phase implied by the line widths observed in the total lipid extracts and the purified phospholipids, the relative amount of fluid lipid seen at any given temperature is the same in these two lipid dispersions. The profile of relative intensity vs. temperature shows a smooth and approximately linear rise through the phase transition. This is exactly the behavior seen in the model pure phospholipid system, but it is significantly different than that in the intact *E. coli* membrane.

The temperature dependence of the line width in the two different lipid dispersions derived from *E. coli* is qualitatively the same as in pure phosphatidylcholine dispersions. There is a gradual decrease in both ν_{DD} and ν_0 with increasing temperature. The temperature dependence is greatest in the transition region but still not large enough to account for the sudden increase in the height of the narrow resonance in this temperature range. Clearly, the temperature dependence of ν_0, seen in the intact *E. coli* membrane, cannot be explained simply in terms of the lipid composition of the membrane.

Discussion

Implication of Results Obtained from Model Membrane Systems. The present ^{19}F NMR results can be compared to those of a multitude of other physical techniques that have been applied to study the phase transition in aqueous dispersions of DPPC (Levine, 1972; Chapman, 1975; Lee, 1977). These results do not quantitatively agree, especially with respect to the magnitude of the change that is attributed to premelting just before the gel to liquid-crystal transition. It is surprising that the single component composition of DPPC would give an ambiguous phase-transition behavior. We wish to comment on the nature of the premelting phenomenon because it can be used to explain the apparent divergence of results, obtained by spectroscopic probes vs. results of studies of bulk properties of the lipid bilayer. The ^{19}F resonance is informative in this respect because the signal that gradually appears between 35 and 40 °C is very different from that seen in the liquid-crystal state of DPPC.

Magnetic resonance studies of other nuclei of the DPPC molecule are most similar to the ^{19}F NMR probe technique. In unsonicated dispersions of DPPC there is little or no detectable sharp ^{13}C or ^{1}H signal due to the fatty acyl chain methylenes below 40 °C (Sheetz and Chan, 1972; Lee et al., 1976). ^{2}H NMR studies of the observed quadrupole splittings of the fatty acyl chain methylene segments show a large change in the order parameter around 41 °C (Seelig and Seelig, 1977). There appears to be a sudden onset of motion of the phospholipids at the main transition that is detected by each nucleus attached to the chain. Above the transition there must be continuous rotation and isomerization motions in order to see the relatively narrow line width or quadrupole splitting observed. In this respect the ^{19}F probe technique agrees with other NMR studies that measure the phospholipid acyl chain motion directly.

If a comparison is made among more disparate techniques than the various magnetic resonance studies, there is less agreement about the effect of the transition on the motion of the phospholipid fatty acyl chains. The techniques used to explore the lipid bilayer phase transition can be divided into those that are sensitive to premelting phenomena and those that are not. Tempo partitioning (Shimshick and McConnell, 1973), chlorophyll fluorescence (Lee, 1975), fluorescence polarization of DPH (Lentz et al., 1976a), and light scattering (Tsong et al., 1977) are some methods that show an effect due to premelting that is greater than 20% of that for the main transition of DPPC. DSC and dilatometry (Hinz and Sturtevant, 1973; Nagle, 1973) are techniques that show only a 10% change due to temperature-dependent phenomena between 35 and 40 °C. Raman spectroscopy, which measures the average behavior of all the lipids rather than that of the dilute probe molecule, also shows some effects due to premelting (Gaber and Peticolas, 1977).

According to the ^{19}F NMR results, some temperature-dependent change in the geometry or molecular motion within the bilayer must occur before the main transition of DPPC at 41 °C. This change does not correspond to fast bond isomerizations about the various carbon–carbon bonds of the fatty acyl chains coupled to fast rotational motion of the phospholipids around the long molecular axis. These phenomena are also ruled out by DSC, dilatometry, and X-ray diffraction measurements, which are more consistent with the retention of a relatively close hexagonal packed arrangement of the phospholipid chains below 41 °C (Nagle, 1973; Chapman, 1975). There is insufficient expansion of the bilayer, absorption of heat, or loss of a sharp X-ray diffraction peak, corresponding to a discrete interchain distance, to explain premelting in terms of continuous isomerizational motion of the fatty acid phospholipid chains.

The gradual premelting phenomenon can be explained in terms of defect regions in the two-dimensional crystal lattice of the phospholipid bilayer. More details of this phenomenon can be found in several recent papers that describe such lipid phase transition associated phenomena (Tsong et al., 1977; Lee, 1977). Thus, a gradual change in the physical or spectroscopic properties of the DPPC bilayer is expected up to 41 °C.

These hypothetical discussions of the phase transition in DPPC multilayer dispersions can be related to the ^{19}F NMR studies and to the other studies mentioned above. The theory of the ^{19}F resonance that we present predicts that a sharp resonance will be observed only if there are regions of phospholipid whose fatty acyl chains are continuously undergoing both rotational motions and bond isomerizations. Such a resonance is only seen above 41 °C. This behavior supports the

well documented fluid state of the DPPC at this temperature. It also rules out any discrete fluid lipids as described above in the temperature range between the pretransition and the main transition.

The broad ^{19}F resonance that gradually appears at the onset of the phase transition does correspond to lipids that undergo rotational or translational motion but no internal isomerizations about the carbon–carbon bonds. The resonance is fit well by a simulated spectrum with a dipolar width, ν_{DD}, of 7.5 kHz and a static width (or ν_0) of 1–2 kHz. This is the limit of the spectral simulation in that the theory described is invalid for fatty acyl chains that do not rotate about the chain axis faster than 10^{-5} s. The rotational motion will narrow the dipolar width to 7.5 kHz. More importantly it will increase the spin–lattice relaxation rate to the point where the resonance is no longer saturated during observation, if the motion occurs fast enough. The intensity of the broad resonance at 41 °C observed does, indeed, correspond to the total intensity observed at much higher temperatures. The behavior of the ^{19}F resonance between 40 and 35 °C can be explained by the presence of crystal defects that move by rotational and translational motions of the phospholipids or a general premelting that causes uniform but extremely restricted motion. As the defects become fewer and migrate slower at lower temperatures, the lipids do not move sufficiently quickly to provide adequate spin–lattice relaxation for the ^{19}F nuclei and the resonance disappears.

The hydrophobic probe molecules, such as DPH, 3-nitroxylcholestane, and Tempo, report on the temperature dependence of DPPC multilayers in a different fashion because they are probably more sensitive to the defect regions present below the main transition. All of these molecules have a tendency to be found in such regions because they are not the same shape as lipids and would locally disrupt close packed crystalline regions of the hydrocarbon chains.

DSC, dilatometry, and other studies of macroscopic properties of DPPC show a distinct pretransition at 35 °C. This distinct event may correspond to the initial creation of defects in the bilayer plane, since the probe techniques discussed above show spectral changes before and up to 35 °C with little further change at that temperature. There is a less noticeable, but nevertheless significant, gradual change in the heat capacity and in the temperature dependence of the molar volume that can be associated with premelting near the main transition (Mabrey and Sturtevant, 1976; Nagle and Wilkinson, 1978). This small and gradual change probably corresponds to an increased defect volume in the bilayer. The development of a ^{19}F resonance to the full intensity seen at high temperature in this temperature range implies that all the phospholipids feel the effect of packing defects and they must migrate within the two-dimensional array of the bilayer membrane.

Studies of the phase transition in the DMPC–DSPC mixed lipid model membrane system suggest that motion due to premelting or the creation of microscopic defects in the packing of the phospholipids at temperatures just below that of the appearance of fluid lipids have a significant effect on the properties of the membrane. This system, which exhibits a broad transition 10–20 °C wide at most compositions, shows motion due to premelting according to the appearance of a broad ^{19}F resonance. Because of the difference in chain length of the fatty acids of the phospholipids, it is possible that the two components will undergo phase separation in binary mixtures (e.g., Op Den Kamp et al., 1975). Studies via several techniques have been used to determine whether such a phase separation occurs or whether "nonideal mixing" of the two components is a more correct view of the system. Phase diagrams have been constructed for the possible combinations of the two lipids that argue the point. By measuring the solubility of the EPR probe, Tempo, in dispersions of DMPC and DSPC, Shimshick and McConnell (1973) have constructed a phase diagram from which they imply that a phase separation occurs. Studies of DPH fluorescence support the conclusion derived from the Tempo results (Lentz et al., 1976b). Mabrey and Sturtevant (1976), on the other hand, have conducted sensitive differential scanning calorimetry measurements in the same system that yield a similar but not identical phase diagram. They argue that no distinct phase separation occurs. The various methods agree on the shape of the solidus–fluidus phase diagram, at the fluidus boundary. However, the solidus boundary has been interpreted differently for the various measurements. Because this system has already been characterized by three different techniques, and yet there is still some debate about the phase transition process, we think that it is useful to discuss the system with reference to the ^{19}F resonance of trace amounts of difluoromyristate incorporated as the reporter group.

The ^{19}F narrow resonance intensity vs. temperature diagrams shown in Figure 6 presumably measure exactly the same aspect of the phase transition as the Tempo partitioning, namely, the proportion of fluid lipid regions present. Both probes show a smooth, monotonic increase in the amount of fluid lipid with temperature through the transition range. There is no indication of a range where the intensity remains constant. There are significant differences between the two probes, however. In pure phospholipids, the Tempo partitioning is almost as sensitive to the pretransition as it is to the main transition, the latter corresponding to the actual gel to liquid-crystalline transition of the lipid bilayer. The ^{19}F NMR method seems to be relatively unaffected by the pretransition and shows no evidence for fluid lipid regions in this temperature range. The temperature at which the transition first starts to occur is uniformly lower when measured by the Tempo spin label relative to the difluoromyristate probe. In this regard it should be noted that in this temperature region the difluoromyristate resonance shows changes quite different from a superposition of broad and narrow resonances discussed above.

The large error bars on the solidus curve of the phase diagram in Figure 7 correspond to the temperature region in which the broad ^{19}F resonance does not correspond to either solid or fluid regions. For the purpose of spectral simulation, it is assumed to arise from frozen regions of the phospholipid bilayer. However, the Tempo results imply that in some aspects the phospholipids are fluid in this temperature range. Using arguments identical to those proposed for the pure DPPC transition, the low temperatures for the first formation of fluid lipids implied by DPH and Tempo probes can be explained entirely in terms of crystal defect regions that grow and diffuse faster, setting the lipids in motion as the temperature is raised. The appearance of a ^{19}F resonance with a width of approximately 7 kHz, due solely to molecular rotation in the absence of conformational isomerization, is consistent with this explanation.

There is closer agreement of the solidus curve in the phase diagram generated from DSC and ^{19}F resonance methods. The onset and completion temperatures of the transition are in agreement for the DMPC–DSPC mixtures that we have studied. The DSC scan shows a variable heat capacity through the transition range with two maxima that presumably correspond to the DMPC- and DSPC-rich regions of the bilayer (Mabrey and Sturtevant, 1976). The ^{19}F resonance of difluoromyristate shows a roughly linear rise in intensity corresponding to a constant rate of melting of the phospholipids.

However, if the DSC curves are integrated, the deviations from a straight line predicted are not great enough to be detected outside the 5% error limits placed on the ^{19}F resonance intensity measurements.

The points on the solidus curve generated by the DSC studies correspond to a sudden increase in heat capacity. These temperatures also correspond to the appearance of a distinct sharp ^{19}F resonance. Below these temperatures there is a measurable excess heat absorption that gradually decreases at lower temperatures. At sufficiently low temperatures a discrete "pretransition" heat absorption is seen. As discussed previously, this pretransition has no detectable effect on the ^{19}F resonance. However, the gradual heat absorption just below the main transition exactly parallels the appearance of the broad "solidlike" ^{19}F resonance.

Thus in DMPC-DSPC mixtures as in pure phosphatidylcholine dispersions, temperature-dependent phenomena are present below the discrete gel to liquid-crystalline transition temperature. In the mixed lipids, the premelting behavior, perhaps corresponding to defect formation and diffusion, is sufficient to set all the phospholipids in motion at a fast enough rate to cause effective spin-lattice relaxation of the ^{19}F resonance. Both DSC and ^{19}F NMR techniques also define a temperature at which truly fluid lipid regions first appear. According to the ^{19}F resonance the molecular motion in the fluid regions is fast and continuous and includes conformational isomerization which distinguishes it from the more restricted motional behavior in the solid.

The solidus-fluidus phase diagram for DMPC-DSPC multilayer dispersions has been used to argue for the presence or absence of a separation of components in the phase-transition region. The Tempo and DPH probes indicate that the solidus curve does not change in temperature from 0 to 50 mol % DPPC, which implies that areas of pure DMPC exist in the solid phase. The solidus curve corresponding to the first appearance of conformationally mobile lipid regions is clearly defined by ^{19}F NMR experiments. This curve does not contain any flat segments and is in general in good agreement with the interpretation of the DSC results. This solidus curve does not correspond to the melting of pure DMPC.

Implication of Results Obtained from E. coli Membranes. A detailed picture of the progress of the phase transition in intact *E. coli* membranes can be obtained from the ^{19}F resonance technique. Because the resonance arises only from biosynthetically incorporated difluoromyristate and is not obscured by any other resonance, unequivocal conclusions can be drawn from these observations (Gent et al., 1978). The most interesting conclusion is that protein plays a large role in modulating the motion of the bulk phospholipids. Three separate types of evidence suggest this. There are dramatic differences in ν_{DD} for the narrow resonance in *E. coli* membranes compared to purified phospholipids or model membranes (Figures 5 and 11). The ultimate narrowing, ν_0, and its temperature dependence are different and more complex than in pure lipid membranes (Figure 10). Finally, the narrow resonance intensity vs. temperature profile, used to define the temperature range of the phase transition, is significantly different for the *E. coli* membrane and lipid extracts thereof (Figure 9).

The relatively broad dipolar line width assigned to the fluid lipid component in *E. coli* membranes suggests that lipid–protein interactions immobilize the lipid hydrocarbon chain-wagging motions. This is especially evident if the resonance of intact membranes is compared to the total lipid extract as shown in Figure 11. The proteins in the membrane more than compensate for the disorder induced by the neutral lipid components. Even though protein-lipid interactions are implied by the broad ν_{DD}, protein denaturation caused by high temperatures does not cause an abrupt narrowing of the resonance.

This influence of membrane protein on the phospholipid motion is like the effect of cholesterol, at least above the phase transition, and it may also be due to geometrical constraints. Membrane proteins are probably rigid, oriented molecules that are inserted into the fluid two-dimensional hydrocarbon phase as is cholesterol. Lipids adjacent to such rigid molecules would have less freedom to flex than those surrounded by other fluid lipids. If lipids in the bulk lipid phase rapidly exchange places with the boundary lipid of the membrane protein, the time-averaged effect would explain the large order parameter seen in the intact *E. coli* membrane (Figure 11).

The ν_0 parameter of the ^{19}F line shape also shows broadening effects, probably due to protein-lipid interactions, but the temperature dependence is more complicated than for ν_{DD}. At temperatures higher than 40 °C, there is a larger decrease in ν_0 in the *E. coli* membrane than in the isolated lipids. At 60 °C, ν_0 is almost the same in the intact membrane and the isolated lipids, suggesting that in this case protein denaturation has eliminated the ordering effect of the protein. Thus, ν_0 seems to be more sensitive to the details of protein-lipid interactions than ν_{DD}. The temperature dependence of ν_0 within the phase transition is also substantially different in the *E. coli* membrane than in lipid extracts. Between 20 and 35 °C the more dramatic narrowing of ν_0 in *E. coli* membranes than in the lipid extracts suggests that there is a decrease in the protein-lipid interaction that affects ν_0. This effect could be due to proportionally more lipid interacting with membrane protein as the temperature increases.

If the ordering effect of the protein on the lipid is a simple geometric constraint, as in the case of cholesterol, the significant change in motion of the fluid lipid component during the transition can be explained. A fundamental change in the distribution of the membrane protein occurs in which proteins are found in clusters below the transition temperature but are randomly dispersed throughout the membrane above the phase transition (Kleeman and McConnell, 1974; van Heerikhuisen et al., 1975). It is likely that the lipid associated with the protein clusters will remain partially fluid. The boundary effects of the high concentration of protein will destroy the cooperative freezing characteristic of the bulk lipid phase. At low temperatures those lipids that are fluid interact with proportionally more proteins than at higher temperature. Thus, the time-averaged ordering effect of the proteins on the fluid lipid would change depending on the degree of clustering. The ^{19}F NMR resonance shows a sharp signal due to a fluid component of about 15% of the total intensity even at 10 °C. This component increases to only 25% at 25 °C, revealing that an approximately constant amount of lipid is involved in the protein clusters and a stoichiometric interaction, of sorts, is involved. This layer of lipids is also known as "boundary" lipids.

One effect of the protein on the phospholipids is quite unlike the effect of cholesterol. The temperature dependence of the sharp resonance intensity in the *E. coli* membrane shows a rise to 100% intensity over a narrower range and at a higher temperature than in membranes of extracted lipids (Figure 9). Cholesterol-like behavior, on the other hand, would be expected to broaden the transition by diminishing the difference between frozen and fluid motional states of all the lipids. Membrane proteins can narrow the temperature range in which the majority of the lipid goes from a frozen to a fluid state suggesting that the protein can influence the lipid not in intimate contact, i.e., that which is not in the clusters of protein visualized by

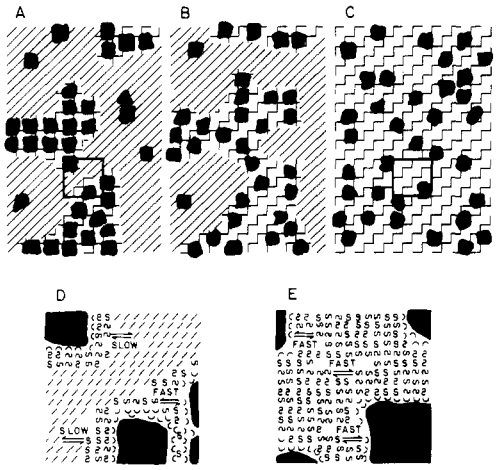

FIGURE 14: A schematic representation of the distribution of lipids and proteins in the plane of the *E. coli* cytochrome membrane. A, B, and C represent temperatures below, within, and above the phase transition, respectively. D and E are expansions of the squares outlined in A and C, respectively, to show the rate of exchange of lipid in various environments: (∪) boundary lipids; (S) fluid lipids; (/) frozen lipids.

electron microscopy. A likely cause for this effect is that the proteins preferentially attract lower melting lipids, leaving the lipid-enriched regions of the membrane with a composition that is more homogeneous and has a higher temperature phase transition. Partial fractionation of lipid has been shown to occur in linoleate-enriched membranes by chemical analysis of separated fractions of lipid- or protein-enriched membrane fragments (van Heerikhuisen et al., 1975).

The complexity of the phase-transition process in the *E. coli* cytoplasmic membrane, compared to model membrane systems or purified lipid extracts, is largely due to the fact that at temperatures below the first formation of clusters of protein there are two populations of phospholipids with very different temperature dependencies. A diagrammatic representation of three stages of the phase transition in an *E. coli* membrane is given in Figure 14 to show the organization of lipid and protein described above. Those lipids that are presumed to associate with the protein remain fluid as defined by the continuous rotation and isomerization motions necessary to give a narrow ^{19}F resonance. The extent of motion of these lipids changes very little from 10 °C until the protein clusters are dispersed at 37 °C. Apparently, the cooperative melting, characteristic of pure lipids, is prevented for lipids in protein clusters due to interactions with the hydrophobic surface of the membrane proteins. The amount of lipid involved in protein clusters stays approximately constant at 15–25% until 25 °C. The rest of the phospholipids are much less affected by protein and undergo a true solid–fluid phase transition.

However, there are two motional states involved in the transition according to the ^{19}F resonance behavior. The first indication of molecular motion in the mostly lipid regions of the *E. coli* membrane is seen in a broad ^{19}F resonance whose width, ν_{DD} = 7.5 kHz, corresponds to lipids which only rotate about the long axis or which exchange with more fluid lipids. These two possibilities allow spin–lattice relaxation which, as discussed earlier, is necessary to see any resonance. The intensity of the broad resonance rises until 25 °C where it corresponds to the full intensity expected for the lipid that is not in the narrow resonance. Above this temperature, the intensity of the broad resonance drops again as the lipids become truly fluid. In lipid extracts there is no wide temperature range in which such a broad resonance appears. For this reason, the broad resonance in the *E. coli* membrane probably corresponds to lipid that is exchanging with the fluid, protein-rich regions, and the lipid is thus in intermittent motion. Above 25 °C, truly fluid lipid regions grow at the expense of the partially mobile lipids. This process continues until all the lipid is in a fluid state and the proteins become randomly dispersed. The process occurring above 25 °C corresponds to the gel to liquid-crystalline transition observed in model membranes. However, below this temperature there is premelting that is much more extended than in homogeneous model membranes, such as DPPC.

Thus, ^{19}F NMR studies of membranes from *E. coli* K1060B5 suggest that proteins play a significant part in the evolution of the membrane phase transition. The amount of fluid lipids at any temperature within the transition is different for intact membranes and for isolated lipids. These statements seem to contradict the conclusions of earlier studies that the phase transition in membranes is the same as in isolated lipids, except for some broadening of the range of the transition (Overath and Trauble, 1973; Jackson and Sturtevant, 1977). Unfortunately, it is hard to make a direct comparison between the present results and earlier studies. In order to incorporate difluoromyristate, the *E. coli* were grown until about 25% of the fatty acids were fluorinated. This gave a heterogeneous mixture of phospholipid fatty acids with very few unsaturates and with no particular fatty acid present to greater than 35%. In contrast, most physical studies of the *E. coli* membrane use unsaturated fatty acid auxotrophs grown under conditions in which 60–90% of the fatty acid is a single unsaturated species. We will discuss our conclusions with those derived from studies of oleate-supplemented cells in which only 50% of the fatty acid is oleate. The midpoint of the transition in these membranes, 10–15 °C, is substantially lower than in fluorinated membranes, 30 °C, but the width of the transition is about the same. X-ray diffraction, Tempo partitioning, and NPN fluorescence studies have been done on both intact membranes and the isolated lipids of the oleate-enriched *E. coli* (Linden et al., 1973; Overath et al., 1975; Thilo et al., 1977).

A fundamental distinction between our phase-transition model and previous hypotheses is that we assume the transition to have a premelting behavior that sets the lipid in partial motion. The conclusion of most other studies is that there is a single melting process with a well-defined beginning and end point. However, there is evidence that different techniques measure different end points for the transition. This phenomenon, also observed in the model membrane system consisting of mixtures of DMPC and DSPC, can be explained in terms of the sensitivity of different methods to the different melting processes.

X-ray diffraction is the most unambiguous method for measuring the extent of phospholipids in hexagonally packed crystalline regions. Studies of the K1060 strain of *E. coli* grown on oleate show that the amount of such frozen lipids decreases linearly from 0 to 20 °C (Overath et al., 1975). NPN fluorescence in the same membrane shows a gradual decrease, presumably due to the disappearance of fluid lipids between 24 and 8 °C (Overath et al., 1975). Further illustrating that more than one transition process takes place, the 30E(β_{ox}-) strain of *E. coli* enriched to 50% with oleate shows breaks in the Tempo solubility parameter at 31 and 16 °C (Linden et al., 1973). In this case it is interesting to note that as the temperature is lowered below 16 °C there is an increasing rate of exclusion of Tempo, a phenomenon that should occur during the phase transition and not at lower temperatures.

One further piece of evidence that suggests that oleate-enriched membranes, like the fluorinated membranes, undergo two distinct melting processes in the phase-transition region

comes from freeze-fracture electron microscopy studies of protein clustering (Kleeman and McConnell, 1974; van Heerikhuisen et al., 1975). The phenomenon is only seen at or below the membrane phase transition and corresponds to a nonrandom distribution of particles on the surface of the *E. coli* cytoplasmic membrane. Areas free of such particles and areas that are densely populated are seen below the transition. Ingenious experiments have shown that the particle-free membranes do correspond to a low protein content (1.74 mg of lipid/mg of protein rather than 0.34 mg of lipid/mg of protein in the whole membrane) and they also correspond to regions of lipid with more saturated fatty acid chains and presumably a higher melting temperature (van Heerikhuisen et al., 1975; Letellier et al., 1977).

The few experiments of this type show that there is no protein clustering at 22 °C but that this phenomenon is complete at 5 °C in oleate-enriched membranes (Kleeman and McConnell, 1974). In eliadate-enriched membranes, in which the process has been more thoroughly studied, protein clustering goes to completion in the temperature range of 41–37 °C, even though several techniques show that the phase transition in this membrane extends from 40 to 28 °C (Kleeman and McConnell, 1974). Thus, in both elaidate- and oleate-enriched cells, protein clustering seems to disappear concomitantly with the high-temperature melting behavior. At lower temperatures the increased amount of crystalline lipids does not lead to further segregation of the protein.

Summary

The ^{19}F resonance of difluoromyristate incorporated into pure phospholipid multilayer dispersions is very sensitive to the gel to liquid-crystalline phase transition. A narrow resonance characteristic of fluid lipid regions can be seen and quantified by spectral simulation, even in the presence of a resonance from frozen lipids. The intensity of the narrow resonance is a true measure of the proportion of fluid lipids present according to comparisons made to the results of other studies of the DMPC–DSPC mixed lipid system. The phase transition in mixed lipid systems corresponds to a gradual increase in the extent of discrete fluid lipid regions while the remaining lipid remains in a rigid or very restricted motional state. Neither fluid nor frozen regions show large changes in their molecular motional properties through the entire range of the transition. No onset of rotational or translational motion of the phospholipid fatty acyl chains is indicated during the pretransition. However, it is evident that very restricted molecular motions occur in the solid regions of the membrane for several degrees below the temperature at which truly fluid lipid domains appear.

The use of spectroscopic probe techniques to investigate the physical state of bilayers in mixed phospholipid systems must be approached with caution. The ^{19}F resonance of difluoromyristate is a sensitive and accurate measure of that proportion of lipids undergoing continuous rotation and bond isomerization motions characteristic of the liquid state. However, few remarks about the nature of the premelting phenomena can be made from studies using this technique. On the other hand, Tempo and DPH probes are so sensitive to premelting phenomena that the first appearance of fluid lipid regions is obscured, at least in broad lipid phase transitions.

The ability of the ^{19}F NMR method to describe the extent of motion of both frozen and fluid lipids simultaneously allows the complex phase-transition behavior in the *E. coli* cytoplasmic membrane to be investigated. All the phase-transition behavior seen in oleate-enriched *E. coli* membranes can also be interpreted in terms of a two-stage melting process used to describe the ^{19}F resonance temperature dependence of fluorinated membranes. The membrane protein has a large effect on the transition behavior of the phospholipids. In both cases, the first appearance of truly fluid lipids does not occur until near the high-temperature end of the phase transition. As fluid lipid regions expand, they disperse membrane proteins in a random fashion in the plane of the membrane. At lower temperatures the membrane proteins are segregated into the fluid lipid regions that remain. However, the nominally frozen lipid continues to undergo some motion. As the membrane is cooled further, the amount of crystalline lipid increases gradually, while the lipid associated with membrane protein remains fluid. At least in the difluoromyristate-enriched membrane, this gradual freezing process continues to temperatures well below the temperature corresponding to complete freezing of extracted lipid membranes. The existence of two different melting phenomena must be taken into account in order to interpret the temperature dependence of spectral probes or of other physical measurements. More importantly, these processes must be quantitatively measured in order to understand the temperature dependence of membrane-associated enzymatic processes such as transport.

Acknowledgment

We thank Dr. David F. Silbert for providing us with the *E. coli* fatty acid auxotroph (K1060B5) used in this work and Dr. R. G. Griffin, Dr. J. F. Nagle, and Dr. D. A. Wilkinson for informative discussions.

Supplementary Material Available

A computer program which simulates the ^{19}F NMR spectra resulting from nonaveraged chemical-shift anisotropy and dipole–dipole interactions. This computer program can be used to generate and display theoretical ^{19}F resonance shapes for given values of the parameters ν_{CSA}, ν_{DD}, and ν_0 (5 pages). Ordering information is given on any current masthead page.

References

Abragam, A. (1961), Principles of Nuclear Magnetism, London, Clarendon Press, Chapter 4.

Baldassare, J. J., Rhienhart, R. C., and Silbert, D. F. (1976) *Biochemistry 15,* 2986.

Bligh, E. G., and Dyer, W. J. (1959), *Can. J. Biochem. Physiol. 37,* 911.

Bloom, M., Burnell, E. E., Roeder, B. W., and Valic, M. I. (1977), *J. Chem. Phys. 66,* 3012.

Chapman, D. (1975), *Q. Rev. Biophys. 8,* 185.

Dadok, J., and Sprecher, R. F. (1974), *J. Magn. Reson. 13,* 243.

Gaber, B. P., and Peticolas, W. L. (1977), *Biochim. Biophys. Acta 465,* 260.

Gent, M. P. N., Armitage, I. M., and Prestegard, J. H. (1976), *J. Am. Chem. Soc. 98,* 3749.

Gent, M. P. N., Cottam, P. C., and Ho, C. (1978), *Proc. Natl. Acad. Sci. U.S.A. 75,* 630.

Gent, M. P. N., and Prestegard, J. H. (1977), *J. Magn. Reson. 25,* 243.

Hinz, H. J., and Sturtevant, J. M. (1972), *J. Biol. Chem. 247,* 6071.

Horowitz, A. F., Horsely, W. J., and Klein, M. P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 590.

Jackson, M. B., and Sturtevant, J. M. (1977), *J. Biol. Chem. 252,* 4749.

Kaback, H. R. (1971), *Methods Enzymol. 22,* 99.

Kantor, H. L., Mabrey, S., Sturtevant, J. M., and Prestegard,

J. H. (1977), *Biochim. Biophys. Acta 466,* 402.
Kleeman, W., and McConnell, H. M. (1974), *Biochim. Biophys. Acta 345,* 220.
Kohler, S. J., and Klein, M. P. (1977), *Biochemistry 16,* 519.
Lee, A. G. (1975), *Biochim. Biophys. Acta 413,* 11.
Lee, A. G. (1977), *Biochemistry 16,* 835.
Lee, A. G., Birdsall, N. J. M., Metcalfe, J. C., Warren, G. B., and Roberts, G. C. K. (1976), *Proc. R. Soc. London, Ser. B 193,* 253.
Lentz, B. R., Barenholz, Y., and Thompson, T. E. (1976a), *Biochemistry 15,* 4521.
Lentz, B. R., Barenholz, Y., and Thompson, T. E. (1976b), *Biochemistry 15,* 4526.
Letellier, L., Moudden, H., and Schechter, E. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 452.
Levine, Y. K. (1972), *Prog. Biophys. Mol. Biol. 24,* 1.
Levine, Y. K., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1972), *Biochemistry 11,* 1416.
Linden, C. D., Blasie, J. K., and Fox, C. F. (1977), *Biochemistry 16,* 1621.
Linden, C. D., Wright, K. L., McConnell, H. M., and Fox, C. F. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2271.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Mabrey, S., and Sturtevant, J. M. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3862.
Mandelkern, L. (1964), Crystalization of Polymers, New York, N.Y., McGraw Hill.
Mathey, F., and Bensoam, J. (1971), *Tetrahedron 27,* 3965.
Mehring, M., Griffin, R. G., and Waugh, J. S. (1971), *J. Chem. Phys. 55,* 746.
Morrisett, J. D., Pownall, H. J., Plumlee, R. T., Smith, L. C., Zehner, Z. E., Esfahani, M., and Wakil, S. J. (1975), *J. Biol. Chem. 250,* 6969.
Nagle, J. F. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3443.
Nagle, J. F., and Wilkinson, D. A. (1978), *Biophys. J.* (in press).
Niederberger, W., and Seelig, J. (1976), *J. Am. Chem. Soc. 98,* 3704.
Op Den Kamp, J. A., Kanerz, M. T., and van Deenen, L. L. M. (1975), *Biochim. Biophys. Acta 406,* 169.
Overath, P., Brenner, M., Gulik-Krzywicki, T., Schechter, E., and Letellier, L. (1975), *Biochim. Biophys. Acta 389,* 358.
Overath, P., and Trauble, H. (1973), *Biochemistry 12,* 2625.
Petersen, N. O., and Chan, S. I. (1977), *Biochemistry 16,* 2757.
Seelig, J., and Niederberger, W. (1974), *J. Am. Chem. Soc. 96,* 2069.
Seelig, A., and Seelig, J. (1977), *Biochemistry 16,* 45.
Sheard, B. (1969), *Nature (London) 233,* 1057.
Sheetz, M. P., and Chan, S. I. (1972), *Biochemistry 11,* 4573.
Shimshick, E. J., and McConnell, H. M. (1973), *Biochemistry 12,* 2351.
Silbert, D. F., Ladenson, R. C., and Honegger, J. L. (1973), *Biochim. Biophys. Acta 311,* 349.
Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. L. (1965), *J. Am. Oil Chem. Soc. 42,* 53.
Stockton, G. W., Polnasek, C. F., Tullock, A. P., Hason, F., and Smith, I. C. P. (1976), *Biochemistry 15,* 955.
Templin, P. R. (1956), *Ind. Eng. Chem. 48,* 154.
Thilo, L., Trauble, H., and Overath, P. (1977), *Biochemistry 16,* 1283.
Trauble, H., and Overath, P. (1973), *Biochim. Biophys. Acta 307,* 491.
Tsong, T. T., Greenberg, M., and Kanehisa, M. I. (1977), *Biochemistry 16,* 3115.
Urbina, J., and Waugh, J. S. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 5062.
van Heerikhuisen, H., Kwak, E., van Brugger, E. F. J., and Witholt, B. (1975), *Biochim. Biophys. Acta 413,* 177.
van Willigen, H., Griffin, R. G., and Haberkorn, R. A. (1977), *J. Chem. Phys. 67,* 5855.
Wennerström, H. (1973), *Chem. Phys. Lett. 18,* 41.