ANALYSIS OF THE COMPOSITION OF MIXED LIPID PHASES BY THE MOMENTS OF ^2H NMR SPECTRA

HAROLD C. JARRELL, R. ANDREW BYRD, AND IAN C. P. SMITH, *Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6*

ABSTRACT The use of ^2H NMR spectral moments to determine the composition of biphasic lipid mixtures is outlined. The analysis has been applied to phosphatidylethanolamine-cholesterol (1:1), potassium palmitate, 30% (wt/wt) water and phosphatidylcholine-cholesterol (4:1) systems, as well as to membrances of *Escherichia coli* during phase transitions. The advantages and disadvantages of the use of spectral moments to determine fractions of coexistent phases are discussed.

INTRODUCTION

Deuterium magnetic resonance studies of model and of natural membranes has resulted in order-position profiles for the acyl chains of lipids in the liquid-crystalline phase (Seelig and Seelig, 1974, 1975; Stockton et al., 1978; Davis et al., 1979*a, b;* Nichol et al., 1980; Smith et al., 1979; Kang et al., 1981) and has yielded information on the effects of protein and cholesterol on order (Oldfield et al., 1976, 1978; Kang et al., 1979, 1981; Rice and Oldfield, 1979; Jacobs and Oldfield, 1979; Stockton and Smith, 1976; Stockton et al., 1976). Recently ^2H spectral moments have been used to interpret the phase behaviour of natural (Nichol et al., 1980; Davis et al., 1979*a, b,* 1980; Smith et al., 1979) and model membranes (Davis, 1979). Although the phase transitions of various model and natural membranes have been investigated extensively, to our knowledge there have been only a few reports of attempts to measure the fractions of the component phases. In the case of *Escherichia coli* membranes, the fractions of fluid and gel phases were estimated from x-ray diffraction data (Overath et al., 1975), and were obtained directly from the ^2H spectrum by taking the ratio of the estimated spectral area of the fluid component to the total spectral area (Nichol et al., 1980). In another study the fraction of fluid phase for *Acholeplasma laidlawii* membranes (Kang et al., 1981) and *E. coli* membranes (Kang et al., 1979) as well as isolated lipids were determined by simulating the broad components of the ^2H spectra. Finally, the moment ratio $[M_4r/(M_2r)^2]$ has been used[1] to obtain information about the proton NMR lineshape of dipalmitoylphosphatidylcholine (DPPC) and the ratio was used to determine the mole fraction of gel-state lipid in multilamellar dispersions of DPPC at 315°K.

The reliability of the above methods has not been indicated or verified. An accurate knowledge of gel- and fluid-phase fractions in a natural membrane would be useful in assessing more quantitatively the effects of protein and cholesterol on lipid organization as

Issued as National Research Council of Canada No. 19004.
[1]MacKay, A. L. 1981. Manuscript submitted for publication.

well as correlating the physical properties of membranes having different fatty acid compositions.

We felt that ^2H spectral moments might afford a means of determining more accurately the relative amounts of the fractions in a two-phase mixture at a given temperature. A method is proposed herein that permits analysis of the fraction of each phase present by deconvolution of the spectral moments of the observed spectra into the component parts representing the contributing phases. The results of this method can be readily evaluated by comparison of the observed spectra with spectra simulated from fractional addition of spectra of the two phases based on the determined values. In an effort to assess the reliability of the method we have used phosphatidylethanolamine-cholesterol (1:1), potassium palmitate—30% (wt/wt) water, and phosphatidylcholine-cholesterol (4:1) as model systems.

We first assume that, inasmuch as gel and liquid-crystalline phases are in slow exchange on the ^2H NMR time scale, the ^2H NMR spectrum in the phase transition region is a simple superposition of gel and fluid state spectra. Thus, in accord with previous workers (Nichol et al., 1980), the n^{th} moment of the ^2H spectrum of a mixed phase is given as

$$M_n = fM_n^L + (1 - f)M_n^G \tag{1}$$

where f is the fraction of lipid in the fluid phase, and M_n^L and M_n^G are the n^{th} moments[2] of the fluid and gel components of the spectrum, respectively. Since M_n^L and M_n^G remain essentially constant for one or two temperature intervals above and below the phase transition, respectively, (see Tables I–IV) we assume initially that M_n^L and M_n^G remain invariant throughout the phase transition. We will later outline a procedure that removes this assumption for M_n^G; the assumption that M_n^L remains constant throughout the phase transition has been used previously (Nichol et al., 1980). In order to assess the accuracy of the calculated fractions, experimental gel- and fluid-state spectra are combined to simulate spectra for a given temperature and then compared with the experimental spectrum at that temperature.

METHODS

Phosphatidylethanolamine-d_4, deuterated in the aminoethyl function, was prepared as outlined by Taylor and Smith (1981). Phosphatidylcholine labeled at the *sn*-2 position with [12,12-d_2] palmitic acid was prepared from egg phosphatidylcholine and was a generous gift of Y. Boulanger (Boulanger, 1980). Lipids were shown to be pure by thin layer chromatography. Potassium [2,2-d_2]-, [6,6-d_2]- and [13,13-d_2] palmitates were prepared by dissolving the acid (Tulloch, 1979) in ethanol, adding one equivalent of potassium hydroxide, removing the solvent and drying under vacuum. Cholesterol and deuterium-depleted water were obtained from Aldrich Chemical Co., Milwaukee, Wis.

---

[2]The n^{th} moment of a spectrum defined by a lineshape function $F(\omega - \omega_0)$, where ω_0 is the center frequency of the symmetric powder pattern, is given by

$$M_n = \frac{\int_0^\infty x^n F(x)dx}{\int_0^\infty F(x)dx}$$

where $x = \omega - \omega_0$. This expression relies on the symmetric nature of the spectrum to yield usable odd and even moments, since integration over the entire pattern would cause the odd moments to vanish.

Potassium palmitate samples were hydrated with deuterium-depleted water (30% by weight) and the warmed mixture (~333°K) was repeatedly centrifuged through a constriction in a glass tube (10-mm o.d.) until the sample looked homogeneous; the sample was then sealed under vacuum. Phosphatidylethanolamine-cholesterol (1:1) and phosphatidylcholine-cholesterol (4:1) samples were prepared by dissolving the lipid and cholesterol in chloroform-methanol (2:1 [vol/vol]) followed by removal of the solvent under a stream of nitrogen, and leaving the sample under vacuum for 24 h. The lipid samples were hydrated with excess deuterium-depleted water, repeatedly vortexed at 318°K, and freeze-thawed; the cycle was repeated until the samples looked homogeneous.

Spectra were obtained at 46.063 MHz on a Bruker CXP-300 spectrometer (Bruker Instruments, Inc. Billerica, Mass.) using a home-built probe. All spectra were obtained using the quadrupolar echo technique (Davis et al., 1976) with phase alternation on the pulse applied along the x-direction. Spectra of the potassium palmitate samples were the result of accumulating 2,000 transients, with 90° pulses of 4.25 μs, a pulse separation of 60 μs, a spectral width of 500 kHz, and a recycle time of 0.5 s. For the phosphatidylcholine-cholesterol (4:1) sample, a pulse separation of 25 μs was used to accumulate 50,000 transients, with a spectral width of 1 MHz, a recycle time of 0.25 s and 45° (2.12 μs) pulses. Spectra of the phosphatidylethanolamine-cholesterol (1:1) sample were obtained using a spectral width of 250 kHz, 90° pulses, accumulating 6,000 transients with recycle time of 0.1 s and echo times of 50 μs. The experiments were done on resonance with the phase of the spectrometer reference signal adjusted to give complete absorption signals without the need for any post-Fourier transform-phase correction (Davis et al., 1976, Byrd and Smith, manuscript in preparation). The sample was enclosed in a glass dewar and the temperature was electronically regulated to within ~ ±0.5°K. After a temperature change the sample was allowed 15 min to come to equilibrium.

Moments were calculated from the centre of the spectrum to the point in the spectrum at which no signal could be detected. The certainty of this point was determined by calculation for 10 successively more distant points and averaging the values obtained. These averages are the values reported. Details of the moment calculation that was used are described elsewhere (Byrd and Smith, manuscript in preparation). Calculations were performed with the Bruker ASPECT 2000 computer, which controls the CXP-300 spectrometer, using real-time software written into the standard Bruker software.

Spectral simulations were obtained using experimental spectra (see Results and Discussion) and the spectral addition features of the standard CXP-300 software.

RESULTS AND DISCUSSION

Phosphatidylethanolamine-d_4: Cholesterol (1:1)

The phosphatidylethanolamine-d_4:cholesterol (1:1) system has been shown to undergo a transition from bilayer to hexagonal phase over the range 310–325°K with a concomitant reduction, by a factor of two, in the quadrupolar splittings of the aminoethyl-d_4 group (Taylor and Smith, 1981). The overlap of the spectra attributable to the aminoethyl deuterons of lipid in both phases (Fig. 1*A*) makes impractical the use of integration to determine the relative amounts of bilayer and hexagonal phase at a given temperature. The values of the first three moments, M_1, M_2, and M_3, for several temperatures throughout the phase transition are given in Table I. The first three moments change very little, <3%, between 298° and 310°K suggesting that the assumption that M_n^G (used in this case to represent the bilayer phase)[3] is essentially constant throughout the phase transition, is reasonable in this case. The slightly larger variation of M_3 between 298° and 310°K is to be expected because of insufficient signal-to-noise (S/N) ratio to provide the higher degree of accuracy required by the higher

[3]In order to maintain consistent notation, the concepts of gel and fluid states, indicated by the G and L superscripts, refer only to the phases below and above, respectively, the phase transition under study.

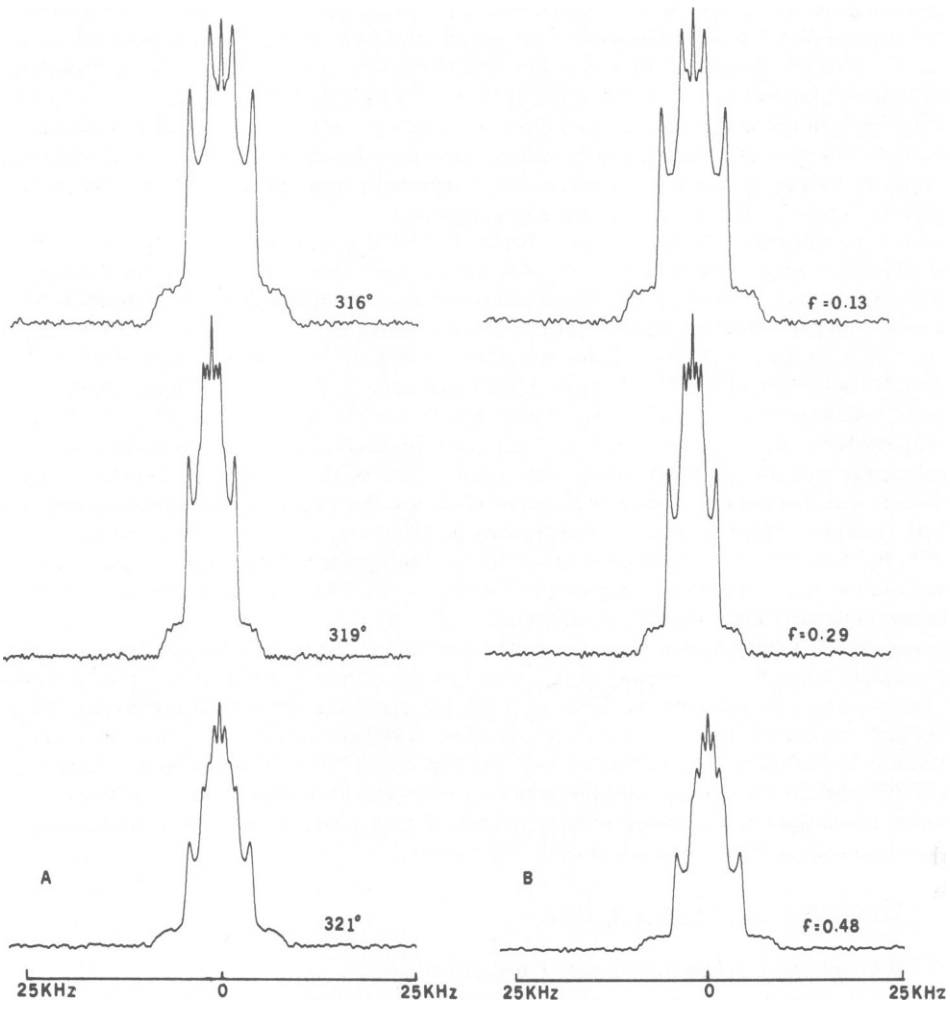

FIGURE 1 (*A*) ^{2}H NMR spectra of phosphatidylethanolamine-d_4:cholesterol (4:1) at 46.063 MHz, after accumulating 6,000 scans using the quadrupolar echo technique, and 90° (4.5 μs) pulses. (*B*) ^{2}H NMR spectra simulated by combining spectra obtained at 326° and 310°K according to the indicated fractions (*f* is for the hexagonal-phase fraction).

moments (Byrd and Smith, manuscript in preparation). Taking M_n^L, the hexagonal phase moments, as the moments of the spectrum at 326°K (when the phase transition is just completed) and M_n^G, the bilayer phase moments, as those of the spectrum at 310°K (where only bilayer is present), the fractions of hexagonal phase (f) were calculated using Eq. 1. The values obtained using M_1, M_2, and M_3 were averaged to give the fractions listed in Table I. The fractions calculated from M_1, M_2, and M_3 differed by ±10% or less when $f \geq 0.15$ and by ~ ±30% when $f \simeq 0.02$.[4] In order to assess the accuracy of the fractions calculated at a given

[4]As a check of internal consistency Eq. 1 can be used to determine the bilayer fraction (i.e., $M_n = (1 - f)\, M_n^L + f M_n^G$). The bilayer fractions so determined are in good agreement with those determined above.

TABLE I

MOMENT DATA AND HEXAGONAL PHASE FRACTIONS FOR THE PHOSPHATIDYL-ETHANOLAMINE-d_4: CHOLESTEROL (1:1) SYSTEM

Temperature	M_1	M_2	M_3	Δ_2	f*
°K	$\times 10^{-4}$ (s^{-1})	$\times 10^{-8}$ (s^{-2})	$\times 10^{-13}$ (s^{-3})		
298	1.74	4.61	1.53	0.132	0.000
310	1.71	4.47	1.47	0.132	0.000
312	1.70	4.43	1.44	0.130	0.016
314	1.67	4.23	1.35	0.137	0.076
316	1.63	4.05	1.26	0.130	0.133
317	1.60	3.97	1.24	0.144	0.159
319	1.50	3.55	1.07	0.171	0.288
320	1.42	3.28	0.988	0.200	0.369
321	1.34	2.92	0.836	0.208	0.482
322	1.25	2.60	0.723	0.231	0.567
324	1.08	1.98	0.512	0.259	0.779
326	0.899	1.25	0.216	0.142	1.000

*f, average fraction of hexagonal phase determined from M_1, M_2, and M_3.

temperature, fractions of the normalized experimental spectra at 310° and 326°K, representing pure bilayer and hexagonal phase, respectively, were added according to the calculated ratios and the resultant spectrum was compared to the experimental spectrum at that temperature (Fig. 1 *B*). The relative peak height ratios of the simulated spectra were measured and compared with the corresponding ratios in the experimental spectrum. The ratios calculated from the simulated spectrum were found to agree with those from the experimental spectrum to within ±5% (Fig. 1 *B*). The average fractions calculated from the first three spectral moments may be taken to be reliable measures of the relative amounts of the phases present at a given temperature for the bilayer-hexagonal phase transition.

The distribution of order parameters has been shown (Nichol et al., 1980) to be reflected in the parameter Δ_2 as given by

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

By substituting Eq. 1 into 2 one can calculate at what fraction of hexagonal phase (f) the Δ_2 parameter will be maximal. We have calculated that Δ_2 should be maximal when f is 0.90, in good agreement with the observed Δ_2 maximum occurring at 324°K where f is 0.78. The latter result offers further support for the assumption of the invariance of both M_n^L and M_n^G throughout the phase transition.

Phosphatidylcholine: Cholesterol (4:1)

Although from the foregoing the spectral moments appear to provide a means of determining phase fractions, we sought a system on which the limitations of the procedure might be stringently tested. The effect of cholesterol on the ordering of egg phosphatidylcholine bilayers has been studied (Stockton et al., 1976) and it was particularly strong at positions 2-12 of stearic acid. Phosphatidylcholine containing cholesterol (4:1) has a phase transition that is ~20° in width (Ladbrooke et al., 1968). In the case of egg phosphatidylcholine which

has been labeled at the *sn*-2 position with [12,12-d_2]-palmitic acid, in the presence of 20 mol% cholesterol, the ^{2}H spectral differences on going from fluid to gel state are minimal (Fig. 2*A*). The first three ^{2}H spectral moments for this system at various temperatures are given in Table II. Whereas in the phosphatidylethanolamine-cholesterol system the calculated moments underwent significant changes on going from one phase to another, the moments of the phosphatidylcholine-cholesterol system exhibit less dramatic changes. Inspection of Fig. 2 *A* reveals that the use of peak integration would be difficult, so that determining phase fractions at various temperatures would be unreliable. The first three moments M_1, M_2, and M_3 were used to calculate fluid phase fractions; the values are given in Table II. M_1 proved to be particularly unreliable because of the small differences involved (note that M_1 varied by a

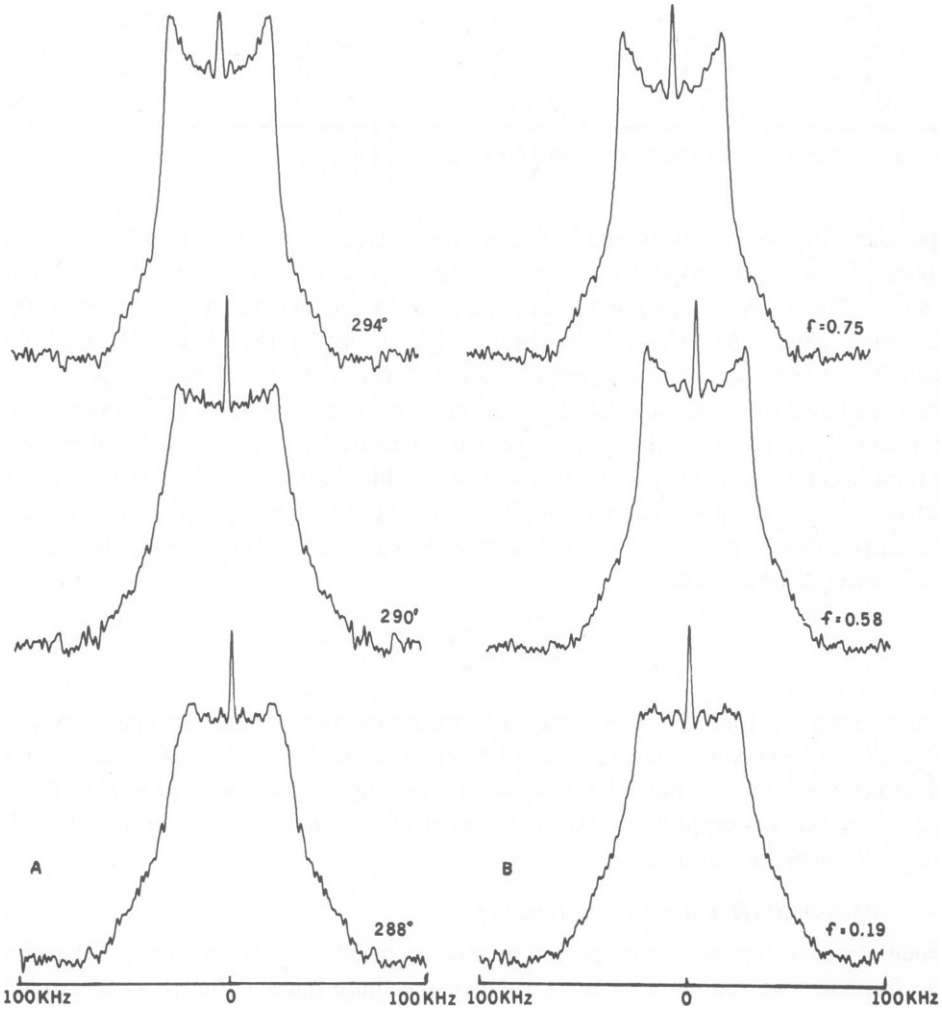

FIGURE 2 (*A*) ^{2}H NMR spectra of phosphatidylcholine-d_2:cholesterol (4:1) after accumulating 50,000 transients using 45° (2.25 μs) pulses and spectral width of 1 MHz. (*B*) ^{2}H NMR spectra simulated by combining spectra obtained at 302° and 382°K according to the indicated fractions (f is for the liquid-crystalline phase fraction).

TABLE II

MOMENT DATA AND FLUID-PHASE FRACTIONS FOR THE PHOSPHATIDYLCHOLINE*: CHOLESTEROL (4:1) SYSTEM

Temperature	M_1	M_2	M_3	Δ_2	f‡
°K	$\times 10^{-5}$ (s^{-1})	$\times 10^{-10}$ (s^{-2})	$\times 10^{-15}$ (s^{-3})		
283	1.42	2.92	7.34	0.0834	0.000
288	1.37	2.77	6.82	0.0853	0.193
290	1.32	2.51	5.73	0.0674	0.578
292	1.29	2.42	5.48	0.0728	0.678
294	1.30	2.38	5.28	0.0526	0.745
298	1.27	2.25	4.78	0.0375	0.925
302	1.26	2.19	4.56	0.0312	1.000
304	1.27	2.24	4.65	0.0231	1.000

*Labeled at the *sn*-2 position with [12,12-d_2] palmitate.

‡ f, average fraction of fluid phase determined from M_1, M_2, and M_3.

factor of 2–6 in the other systems discussed herein). The accuracy of the calculated fractions was assessed by the addition of fractions of normalized experimental spectra (288° and 302°K), and the resultant spectra were compared with the experimental spectrum at a given temperature (Fig. 2 *B*). We are able to detect noticeable discrepancies between simulated and experimental spectra when errors of ±15% in the fractions are included. We feel that the accuracy is substantially less than that for the other examples discussed in this study, but is of the order of ±15%. It appears that when the spectral characteristics of the two phases are similar, moments can provide a means of determining phase fractions, but with a concomitant loss of accuracy.

The phosphatidylethanolamine and phosphatidylcholine systems analyzed above represent two cases where the integration method (Nichol et al., 1980) of determining the fractional composition of the system at a given temperature is not feasible. The present method offers a practical solution to the problem.

TABLE III

MOMENT DATA AND FLUID-PHASE FRACTIONS FOR THE POTASSIUM [6,6-d_2]- AND [13,13-d_2] PALMITATES: 30% (wt/wt) H_2O SYSTEM

Temperature	M_1	M_2	M_3	Δ_2	f*
°K	$\times 10^{-5}$ (s^{-1})	$\times 10^{-10}$ (s^{-2})	$\times 10^{-15}$ (s^{-3})		
298	1.36	2.84	7.12	0.140	0.000
300	1.36	2.79	6.82	0.122	0.000
301	1.37	2.86	7.11	0.127	0.000
302	1.33	2.73	6.69	0.141	0.020
303	1.27	2.56	6.18	0.168	0.090
304	1.22	2.45	5.98	0.214	0.123
305	1.13	2.22	5.41	0.297	0.209
306	0.970	1.84	4.47	0.448	0.349
307	0.799	1.44	3.49	0.668	0.497
308	0.617	1.00	2.43	0.953	0.656
310	0.267	0.102	0.0489	0.0641	1.000
312	0.266	0.103	0.0491	0.0700	1.000

*f, average fraction of fluid phase determined from M_1, M_2, and M_3.

TABLE IV
MOMENT DATA AND FLUID-PHASE FRACTIONS FOR THE POTASSIUM [13,13-d_2] PALMITATE: 30% (wt/wt) H_2O SYSTEM

Temperature	M_1	M_2	M_3	Δ_2	f*
°K	$\times 10^{-5}$ (s^{-1})	$\times 10^{-10}$ (s^{-2})	$\times 10^{-15}$ (s^{-3})		
288	1.39	2.83	6.93	0.0891	0.000
292	1.39	2.87	7.11	0.0989	0.000
296	1.38	2.89	7.24	0.119	0.000
298	1.33	2.75	6.82	0.154	0.049
300	1.29	2.73	6.95	0.215	0.068
301	1.23	2.59	6.55	0.274	0.120
302	1.14	2.34	5.76	0.338	0.203
303	1.07	2.24	5.75	0.445	0.248
304	0.886	1.75	4.43	0.651	0.406
305	0.762	1.49	3.93	0.901	0.496
306	0.557	0.989	2.66	1.36	0.689
307	0.231	0.0667	0.0245	0.0370	0.990
312	0.214	0.0640	0.0231	0.0386	1.000

*f, average fraction of fluid phase determined from M_1, M_2, and M_3.

Potassium Palmitate—30% (wt/wt) H_2O

The potassium palmitate-water system has been previously studied by ^2H NMR (Davis and Jeffrey, 1977) and provides a convenient system for analyzing gel and liquid-crystalline phases. In the latter study positions 6 and 13 of the palmitate moiety were reported to give rise to different order parameters in the liquid-crystalline phase. As a further test of our proposed method, we have investigated three potassium palmitate—30% (wt/wt) water systems: palmitate labeled at position 2, labeled at position 13, and a 1:1 mixture of palmitate labeled at positions 6 and 13. We calculated the first three moments for spectra throughout the phase transitions and, using Eq. 1, as discussed above, the fractions of fluid phase (f). The results are given in Tables III–V. Because of the difficulty in obtaining identical systems with these soaps (Davis and Jeffrey, 1977) the phase transitions occur at slightly different temperatures; (note that the Δ_2 parameters are maximal at different temperatures for each of the systems). In addition, because the thermal behaviour of these systems is strongly dependent upon the

TABLE V
MOMENT DATA AND FLUID-PHASE FRACTIONS FOR THE POTASSIUM [2,2-d_2] PALMITATE: 30% (wt/wt) H_2O SYSTEM

Temperature	M_1	M_2	M_3	Δ_2	f*
(°K)	$\times 10^{-5}$ (s^{-1})	$\times 10^{-10}$ (s^{-2})	$\times 10^{-15}$ (s^{-3})		
288	1.07	1.65	3.03	0.0634	0.000
292	0.957	1.48	2.64	0.105	0.139
296	0.929	1.32	2.33	0.137	0.225
300	0.766	0.957	1.53	0.206	0.478
304	0.502	0.430	0.496	0.265	0.846
308	0.379	0.198	0.127	0.228	1.000
312	0.370	0.187	0.113	0.0102	1.000

*f, average fraction of fluid phase determined from M_1, M_2, and M_3.

water content and homogeneity of the system, the rate of change of the relative proportions of the phases with temperature may be different in the three preparations. We calculate, as described above, that for the mixture of [6,6-d_2]- and [13,13-d_2] palmitates Δ_2 should be a maximum at $f = 0.64$, in excellent agreement with an observed maximum at $f = 0.66$. Similarly, for the 13-d_2-labeled system, Δ_2 is calculated to be maximal when $f = 0.72$, in good agreement with the observed maximum at $f = 0.69$. The agreement between the calculated and observed fractions at which Δ_2 is maximal suggests that the assumption of temperature invariance for M_n^L and M_n^G throughout the phase transition is reasonable. This assumption is supported further by the observation (Tables III and IV) that the spectral moments M_n^L and M_n^G remain essentially constant above and below the phase transition, respectively, for a few temperature intervals.

The fraction of fluid phase calculated from M_1, M_2, and M_3 agree to within ±5% for $f \geq 0.10$ in the case of of the mixture of [6,6-d_2]- and [13,13-d_2] palmitates, while the agreement decreased to ~ ±10% for the [13,13-d_2]- and [2,2-d_2] palmitate cases. To assess the accuracy of the calculated fractions, the experimental liquid-crystalline-and gel-state spectra were combined as described above (Fig. 3 *B*). The stimulated spectrum was compared with the experimental spectrum at a given temperature by using relative peak height ratios. Calculated spectra were found to agree with the experimental spectra to within ±10%.

An Approach to the Simultaneous Determination of f and M_n^G

Inspection of Tables III and IV reveals that the mixture of [6,6-d_2]- and [13,13-d_2] palmitates gives rise to gel-state spectral moments that have the same values as those observed for the [13,13-d_2] palmitate system. The latter result indicates that positions 6 and 13 of the palmitate chain give rise to the same gel-state spectra just below the phase transition. One might speculate that such a situation may exist in membrane systems so that a more general approach to calculating phase fractions at a given temperature may be pursued in the following way: The moments M_n^L associated with fluid-phase spectra are assumed to be invariant during the phase transition. On the basis of the above observation, the gel-state spectral moments of CD_2 groups at all positions (other than the penultimate CD_2) are assumed to be the same throughout the phase transition. It is thus straightforward to derive from Eq. 1 an expression involving the fraction f, for a system which may be labeled in two positions, that is independent of M_n^G:

$$(M_n)_1 - (M_n)_2 = f[(M_n^L)_1 - (M_n^L)_2], \tag{3}$$

where $(M_n)_i$, $(M_n^L)_i$ are the observed moments (at a given temperature) and the fluid-state moments for label i, respectively. A second term involving the difference in M_n^G for the two labels has been omitted from Eq. 3 based on the previous observations. Thus, for a lipid system in which one has data for two labeled positions for which the $(M_n^L)_i$ are different, it is possible to determine f, the fluid fraction, and hence the gel fraction, as well as the gel-state moment, M_n^G, at a given temperature by application of Eqs. 1 and 3.

Support for this approach may be obtained by applying the analysis to the data reported for *E. coli* (Davis et al., 1979; Nichol et al., 1980). In these studies *E. coli* were grown on [13,13-d_2] palmitic acid plus oleic acid and on d_{31}-palmitic acid plus oleic acid. Below the phase transition (at 278°K) both the cytoplasmic and outer membranes give rise to the same

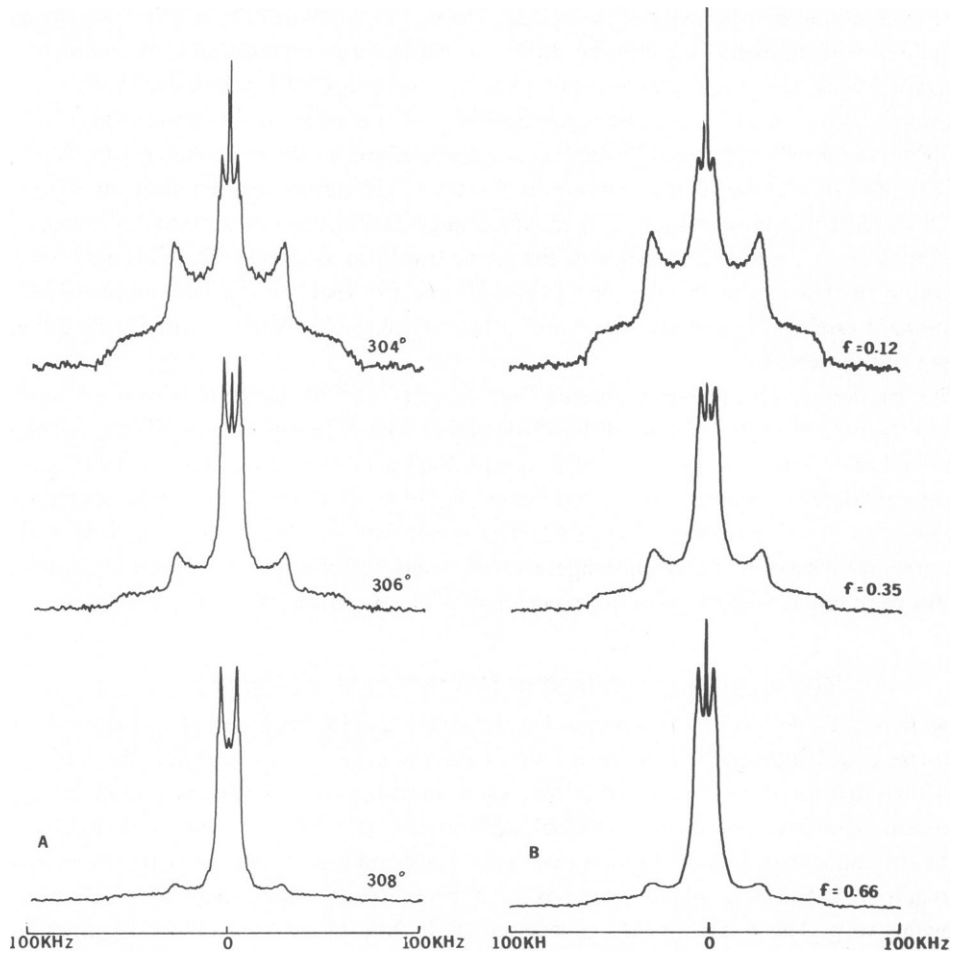

FIGURE 3 (*A*) ^{2}H NMR spectra of a 1:1 mixture of potassium [6,6-d_2]- and [13,13-d_2] palmitates- 30% H_2O at 46.063 MHz, after accumulating 2,000 scans using the quadrupolar echo technique, 90° (4.5 μs) pulses and a spectral width of 250 KHz. (*B*) ^{2}H NMR spectra simulated by combining spectra obtained at 310° and 301°K according to the indicated fractions (*f* is for the fluid-phase fraction).

value of the second moment; both the system containing the 13-d_2-label and that containing the d_{31}-label exhibited the same property. Furthermore, the gel state moments observed for both labels are equal ($\sim 1.75 \times 10^{10}\ s^{-2}$), within experimental error. The latter result supports the hypothesis that M_n^G for CD_2 groups of saturated fatty acyl chains (other than the penultimate position) are essentially equal for a given system in the region of the phase transition. We have applied Eq. 1 to the moment data for [13,13-d_2] palmitate-labeled *E. coli* membranes, and calculated fluid and gel-state fractions which were in general agreement with those reported as measured from the estimated ^{2}H spectral areas (Nichol et al., 1980). The fatty acid composition of membranes containing the 13-d_2-label was essentially the same as that observed for the corresponding membranes containing the perdeutero label. Although the phase transition of the outer membranes containing the 13-d_2-label differed from that of outer membranes containing the d_{31}-label by ~9°, the phase diagrams for the two systems

should be very similar. Assuming that M_2^G was the same for both labels in the outer membrane, Eq. 3 was used to calculate the fluid fraction at two temperatures, i.e., t_c and t_c + 5°K, where t_c is the temperature at which Δ_2 is maximal. At t_c the fluid fraction was calculated to be 0.57 and M_2^G was $1.73 \times 10^{10}\ s^{-2}$, while at t_c + 5°K we calculate a fluid fraction of 0.80 and a M_2^G of $1.84 \times 10^{10} s^{-2}$. Using Eq. 1, we calculate, for the 13-d_2-labeled outer membrane, a fraction of 0.50 at t_c (298°K) and a value of 0.80 at 303°K. These values are in good agreement with the fractions calculated using Eq. 3, whereas fractions of 0.35 (298°K) and 0.75 (303°K) were estimated from spectral areas (Nichol et al., 1980). The gel-state moment, M_2^G, calculated using Eq. 3 is in good agreement with the reported value of $1.63 \pm 0.24 \times 10^{10} s^{-2}$ (Nichol et al., 1980). Furthermore, M_1^G and M_2^G have been reported to be (within an uncertainty of ±10%) independent of temperature throughout the phase transitions for the *E. coli* outer and cytoplasmic membranes (Nichol et al., 1980). These results indicate that the use of spectral moments affords a means of calculating fractions of coexistent phases with an accuracy which is at least as good as that associated with the use of spectral areas; in cases where areas would be difficult to measure, the present method is superior.

A potential limitation of the approach utilizing Eq. 3 becomes evident when the analysis is applied to the potassium palmitate system. Although the 13-d_2-labeled system gives rise to the same value of the gel-state moment as the mixture of [13,13-d_2] and [6,6-d_2] palmitates, application of Eq. 3 fails when moments are used for these systems at temperatures for which f was <0.50. The failure of the analysis may be explained in the following way. If the fluid fractions for the two systems were not exactly equal and differed by, say, 0.02 then Eq. 3 becomes

$$(M_n)_1 - (M_n)_2 = f[(M_n^L)_1 - (M_n^L)_2] + 0.02\,(M_n^L)_1 - 0.02\,M_n^G \tag{4}$$

where $(M_n)_1$, $(M_n^L)_1$ are the moments for label 1 and $(M_n)_2$, $(M_n^L)_2$ are the moments for label 2. For the difference in the fractions to have no effect on the analysis the following inequality must hold

$$|f[(M_n^L)_2 - (M_n^L)_1]| \gg |0.02\,(M_n^L)_2 + 0.02 M_n^G|. \tag{5}$$

For the palmitate system, it is readily shown that Eq. 3 holds only if f is much larger than 0.5. In general, careful selection of the positions of the acyl chain which are labeled (to ensure that the associated M_n^L are significantly different) will allow Eq. 4 to be used to determine f and M_n^G.

A second proviso concerns equality of the M_n^G for the two positions. Since the M_n^G are generally larger than the M_n^L, slight differences in M_n^G will cause large errors in the derived value of f, especially for small values of f. The equality of the M_n^G can be verified by obtaining high quality spectra just below the temperature of the transition.

CONCLUSIONS

The use of ^2H NMR spectral moments affords a reliable method of calculating relative fractions of fluid- and gel-phase lipid in model and natural membranes. Two methods have been described, which may be used separately or together to provide a higher confidence level. The first assumes that the moments of both phases remain constant over the temperature

range of the transition. This appears to be a reasonable assumption if the range is relatively narrow (10–15°K). However, in some systems the phase transition can be broad, such as is observed for *A. laidlawii* membranes containing oleic acid which covers the range 241° to 283°K (Rance et al., 1980). In such cases the temperature invariance of M_n^L and M_n^G may not apply. The second method makes use of the difference in the spectral moments for two labeled positions in the fluid phase, and it assumes that the moments for these two positions are equal in the gel phase throughout the phase transition. The latter assumption has been discussed for the systems presented; however, caution should be exercised when applying this method to a system for which there is little knowledge of its phase behavior. In this case, the assumption may be verified by measurement of the gel-state moments at temperatures just below the phase transition as is inherent in the first method described. Furthermore, care should be taken to ensure that the repetition time of the data acquisition in signal averaging is sufficiently long relative to the spin-lattice relaxation times of the system being studied to yield quantitative spectra of both phases.

Received for publication 19 August 1980 and in revised form 20 January 1981.

REFERENCES

Boulanger, Y. 1980. Ph.D. Thesis. University of Ottawa, Canada.

Davis, J. H., K. R. Jeffrey, M. Bloom, M. I. Valic, and T. P. Higgs. 1976. Quadrupolar echo deuteron magnetic resonance spectroscopy in ordered hydrocarbon chains. *Chem. Phys. Lett.* 42:390–394.

Davis, J. H., and K. R. Jeffrey. 1977. The temperature dependence of chain disorder in potassium palmitate-water. A deuterium NMR study. *Chem. Phys. Lipids.* 20:87–104.

Davis, J. H. 1979. Deuterium magnetic resonance study of the gel and liquid crystalline phases of dipalmitoyl phosphatidylcholine. *Biophys. J.* 27:339–358.

Davis, J. H., B. Maraviglia, G. Weeks, and D. V. Godin. 1979*a*. Bilayer rigidity of the erythrocyte membrane ^{2}H NMR of a perdeuterated palmitic acid probe. *Biochim. Biophys. Acta.* 550:362–366.

Davis, J. H., C. P. Nichol, G. Weeks, and M. Bloom. 1979*b*. Study of the cytoplasmic and outer membranes of *Escherichia coli* by deuterium magnetic resonance. *Biochemistry.* 18:2103–2112.

Davis, J. H., M. Bloom, K. W. Butler, and I. C. P. Smith. 1980. The temperature dependence of molecular order and the influence of cholesterol in *Acholeplasma laidlawii* membranes. *Biochim. Biophys. Acta.* 597:477–491.

Jacobs, R., and E. Oldfield. 1979. Deuterium nuclear magnetic resonance investigation of dimyristoyl lecithin—dipalmitoyl lecithin and dimyristoyl lecithin—cholesterol mixtures. *Biochemistry.* 18:3280–3285.

Kang, S. Y., H. S. Gutowsky, and E. Oldfield. 1979. Spectroscopic studies of specifically-deuterated labelled membrane systems. Nuclear magnetic resonance investigation of protein-lipid interactions in *Escherichia coli* membranes. *Biochemistry.* 18:3268–3272.

Kang, S. Y., R. Hinsey, S. Rajan, H. S. Gutowsky, M. G. Gabridge, and E. Oldfield. 1981. Protein-lipid interactions in biological and model membrane systems. Deuterium NMR of *Acholeplasma laidlawii* B, *Escherichia coli* and cytochrome oxidase systems containing specifically deuterated lipids. *J. Biol. Chem.* 256:1155–1159.

Ladbrooke, H. D., R. M. Williams, and D. Chapman. 1968. Studies on lecithin-cholesterol-water interactions by differential scanning calorimetry and x-ray diffraction. *Biochim. Biophys. Acta.* 150:333–340.

Nichol C. P., J. H. Davis, G. Weeks, and M. Bloom. 1980. Quantitative study of the fluidity of *Escherichia coli* membranes using deuterium magnetic resonance. *Biochemistry.* 19:451–457.

Oldfield, E., M. Meadows, D. Rice, and R. Jacobs. 1978. Spectroscopic studies of specifically deuterium-labelled membrane systems. Nuclear magnetic resonance investigation of the effects of cholesterol in model systems. *Biochemistry.* 17:2727–2740.

Oldfield, E., R. Gilmore, M. Glazer, H. S. Gutowsky, J. C. Hshung, S. Y. Kang, T. E. King, M. Meadows, and D. Rice. 1978. Deuterium nuclear magnetic resonance investigation of the effects of proteins and polypeptides on hydrocarbon chain order in model membrane systems. *Proc. Natl. Acad. Sci. U. S. A.* 75:4657–4660.

Overath, P., M. Brenner, T. Gulik-Kryzwicki, E. Schechter, and L. Letellier. 1975. Lipid phase transitions in cytoplasmic and outer membranes of *Escherichia coli*. *Biochim. Biophys. Acta.* 389:358–369.

Rance, M., K. R. Jeffrey, A. P. Tulloch, K. W. Butler, and I. C. P. Smith. 1980. Orientational order of unsaturated

lipids in the membranes of *Acholeplasma laidlawii* as observed by ^{2}H NMR. *Biochim. Biophys. Acta.* 600:245–262.

Rice, D., and E. Oldfield. 1979. Deuterium nuclear magentic resonance studies of the interaction between dimyristoyl phosphatidylcholine and gramicidin A. *Biochemistry.* 18:3272–3279.

Seelig, A., and J. Seelig. 1974. The dynamic structure of fatty acyl chains in a phospholipid bilayer measured by deuterium magnetic resonance. *Biochemistry.* 13:4839–4845.

Seelig, A., and J. Seelig. 1975. Bilayers of dipalmitoyl-3-*sn*-phosphatidylcholine conformational differences between fatty acyl chains. *Biochim. Biophys. Acta.* 406:1–5.

Smith, I. C. P., K. W. Butler, A. P. Tulloch, J. H. Davis, and M. Bloom. 1979. The properties of gel state lipid in membranes of *Acholeplasma laidlawii* as observed by deuterium magnetic resonance. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 100:57–61.

Stockton, G. W., and I. C. P. Smith. 1976. A deuterium nuclear magnetic resonance study of the condensing effect of cholesterol on egg phosphatidylcholine bilayer membranes. I. Perdeuterated fatty acid probes. *Chem. Phys. Lipids.* 17:251–263.

Stockton, G. W., C. F. Polnaszek, A. P. Tulloch, F. Hasan, and I. C. P. Smith. 1976. Molecular motion and order in single bilayer vesicles and multilamellar dispersions of egg lecithin and lecithin–cholesterol mixtures. A deuterium nuclear magnetic resonance study of specifically-labelled lipids. *Biochemistry.* 15:954–966.

Taylor, M. G., and I. C. P. Smith. 1981. A comparison of spin probe ESR, ^{2}H and ^{31}P NMR for the study of hexagonal phase lipids. *Chem. Phys. Lipids.* 28:119–136.

Tulloch, A. P. 1979. Synthesis of deuterium and carbon-13 labelled lipids. *Chem. Phys. Lipids.* 24:391–406.