^{31}P and ^{2}H NMR Studies of Structure and Motion in Bilayers of Phosphatidylcholine and Phosphatidylethanolamine

Robin Ghosh[‡]

Biocentre, Klingelbergstrasse 70, CH-4055 Basel, Switzerland

Received December 10, 1987; Revised Manuscript Received May 3, 1988

ABSTRACT: The structural and motional properties of mixed bilayers of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) have been examined by using wide-line ^{31}P, ^{14}N, and ^{2}H NMR. ^{2}H and ^{14}N NMR data showed that in mixed bilayers containing both PC and PE the conformations of the head-group moieties are essentially identical with those observed for bilayers containing a single phospholipid species. Equimolar amounts of cholesterol induce also only a small change in head-group conformation. ^{31}P T_1 relaxation measurements (at 300 MHz) at various temperatures of bilayers containing phospholipids with a mixture of phosphocholine and phosphoethanolamine head-groups and unsaturated fatty acid residues revealed in all cases a clearly defined minimum corresponding to the condition $\omega_0\tau_c^{-1} \sim 1$. For all phospholipid mixtures studied, the ^{31}P T_1 relaxation was homogeneous over the whole powder spectrum and could be fitted to a single-exponential decay. The ^{31}P vs temperature profiles were analyzed by a simple correlation model following the analysis of Seelig et al. (1981) [Seelig, J., Tamm, L., Hymel, L., & Fleischer, S. (1981) *Biochemistry 20*, 3922–3932]. Rotational diffusion of the phosphate moiety in bilayers of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was slower than that of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and the activation energy was increased by a factor of 1.7 to 31.4 kJ mol^{-1}. The presence of equimolar amounts of PE containing either the same (POPE) or a different (*Escherichia coli* PE) fatty acid composition had essentially no effect on the rate of rotational diffusion of the phosphate groups, with the correlation time being found to be 0.68 ns at 20 °C. In POPC/POPE bilayers containing 80% POPE, the correlation time was increased to 0.92 ns at 20 °C although the activation energy remained almost constant. In bilayers of *E. coli* PE alone, the correlation time of the phosphate moiety was increased further at 20 °C to 1.65 ns, probably due to extensive formation of intermolecular H-bonding interactions, although the activation energy remained unchanged. The presence of equimolar amounts of cholesterol decreased the correlation time to 0.65 ns, and also the activation energy was reduced to 22.6 kJ mol^{-1}. We interpret the decrease in activation energy as being due to the "spacing" effect of cholesterol which reduces the H-bonding interactions between head-groups, allowing them to rotate more freely. For all cases examined, the rotational diffusion of the phosphate moieties was slower than that observed for the rigid glycerol backbone of the molecule, the latter probably corresponding to overall phospholipid rotation.

The application of magnetic resonance techniques to the study of structure and motion in phospholipids has become increasingly popular in recent years. In particular, ^{31}P NMR has been widely used to investigate the behavior of phospholipid head-groups in pure lipids and reconstituted membranes [Seelig & Gally, 1976; Cullis & DeKruiff, 1976, 1979; Rice et al., 1979; Seelig et al., 1981; Bienvenue et al., 1982; for a review, see Seelig (1978)]. ^{31}P NMR is very attractive as a probe of phospholipid head-groups for several reasons: no labeling is required; the sensitivity is relatively high; and no special pulse techniques are required to obtain good signals. Set against these is the disadvantage that true structural information is difficult to obtain as the chemical shielding anisotropy tensor contains two order parameters whereas only one experimental parameter is available (Seelig & Gally, 1976; Skarjune & Oldfield, 1979).

Recently, ^{31}P T_1 relaxation measurements have been used to examine the motional behavior of phospholipid head-groups in the presence of membrane proteins in reconstituted membranes (Seelig et al., 1981; Tamm & Seelig, 1983). Thus, the calcium pump protein from sarcoplasmic reticulum and mitochondrial cytochrome oxidase were reconstituted into liposomes of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)[1] and 1-palmitoyl-2-oleoyl-*sn*-glycero-o-phosphocholine (POPC), respectively, and in both cases, a minimum in the ^{31}P T_1 vs temperature profile was observed at 121.46 MHz in the presence and absence of protein. The observation of a T_1 minimum allowed Seelig et al. (1981) to obtain a set of correlation times for liposomes of DOPC at various temperatures and a corresponding activation energy. The absence of low-field data for the reconstituted membrane prohibited a detailed analysis of this system.

Several difficulties with this type of analysis still remain. First, low-field data (where the dipole–dipole interaction dominates the relaxation behavior) are necessary to extract a range of correlation times from the ^{31}P T_1 vs T profile. Often this requirement cannot be satisfied due to limitations of time and technical difficulty. Second, the use of a single correlation time model is somewhat controversial. For instance, Brown (1984a) has suggested a two-correlation time model to be the most suitable for the analysis of ^{13}C T_1 relaxation data of

[‡] Present address: Institute for Plant Biology, Zollikerstrasse 107, CH-8008 Zurich, Switzerland.

[1] Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; DSC, differential scanning calorimetry; the molar ratios of the phospholipid mixtures described in the text are given in parentheses.

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phospholipid fatty acyl chains. Finally, the physical significance of the correlation times obtained by Seelig et al. (1981) is still unclear.

This study extends the analysis of Seelig et al. (1981) by means of a simple model, allowing correlation times to be extracted from ^{31}P T_1 vs T measurements made at 121.46 MHz in the absence of the corresponding low-field data. It is demonstrated that at least for the ^{31}P T_1 relaxation of phospholipids in the liquid-crystalline phase that a single correlation time is adequate to describe the data. Finally, we have extended the model to examine the head-group motional behavior of mixtures of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cholesterol. It is shown that in bilayers of PC/PE in the liquid-crystalline phase that the head-groups of both phospholipid species interact with each other such that the rate of head-group rotation of each species is essentially identical.

Materials and Methods

POPC, POPE, and cholesterol were obtained from Berchtold (Bern) *Escherichia coli* [3,3-*glycerol*-^{2}H]-PE was purified as described previously (Gally et al., 1981). Purity of the lipids was checked routinely by using thin-layer chromatography with Merck silica gel plates and $CHCl_3/MeOH/H_2O$ [85/30/3 (v/v)] as the solvent system.

Sample Preparation. Pure lipids and lipid mixtures were dissolved in CH_2Cl_2 and evacuated under vacuum to leave a thin film on a glass tube. The tube was then evacuated under high vacuum for 24 h and subsequently hydrated with 20 mM Tris-HCl, pH 7.5, in deuterium-depleted water, using a vortex mixer. The hydrated lipid was then centrifuged into the NMR tube and measured immediately. Samples were quite stable for 1 month at 2–4 °C and could be frozen and rethawed repeatedly to give identical ^{31}P and ^2H spectra and T_1 relaxation times. After rethawing rapidly, it was necessary to let the sample equilibrate above the phase transition temperature (T_c) for at least 1 h to obtain reproducible NMR measurements.

NMR Methods. NMR measurements were performed with a Bruker Spectrospin CXP-300 spectrometer equipped with a high-power decoupler. ^2H NMR spectra were obtained at 46.06 MHz using a quadrupole echo sequence (Davis et al., 1976). 90° pulses were usually 3–4 μs and the echo pulse separation was 30–40 μs.

Spectra were recorded using quadrature phase detection, and the spectral width was usually 100 kHz. ^2H T_1 relaxation times were measured by using the sequence $[180_x\text{–}t\text{–}90_x\text{–}t_e\text{–}90_y\text{–}t_e\text{–AQ}]_n$ where t_e is the constant quadrupole echo pulse spacing and t is a variable.

^{31}P NMR spectra were recorded at 121.46 MHz using inverse gated decoupling. All spectra were taken using 90° pulses (approximately 3 μs) with a relaxation delay of 8–10 s (approximately 5 times T_1). ^{31}P T_1 relaxation times were taken by using the $[180_x\text{–}t\text{–}90_x\text{–AQ}]_n$ sequence with or without decoupling.

^{14}N NMR spectra were taken at 21.68 MHz using the quadrupole echo technique. The 90° pulse was approximately 10 μs, and the echo pulse spacing was 180 μs with a relaxation delay of 200 ms, using a spectral width of 250 kHz. All NMR experiments employed a phase-alternated sequence to reduce coherent noise and phase errors.

Before each measurement, samples were allowed to equilibrate at the appropriate temperature for at least 30–40 min. Significantly shorter times were not sufficient for adequate temperature equilibration and led to large errors in the measured values of ^2H and ^{31}P T_1 relaxation times. The probe head was routinely tuned before each measurement when necessary.

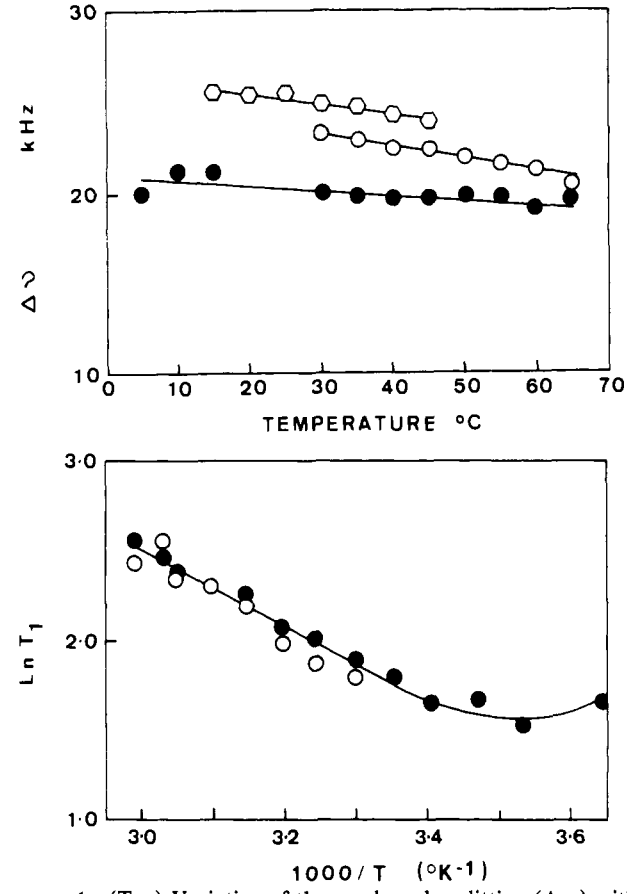

FIGURE 1: (Top) Variation of the quadrupole splitting ($\Delta\nu_Q$) with temperature for bilayers of [3,3-^{2}H]-PE from *E. coli* in the absence (○) and presence (●) of 50% cholesterol [see Ghosh and Seelig (1982) for the original spectra] and bilayers of equimolar POPC/3,3-^{2}H-labeled *E. coli* PE (○). (Bottom) Variation of the ^2H T_1 relaxation time observed for liposomes of 3,3-^{2}H-labeled *E. coli* PE with temperature in the absence (○) and the presence (●) of 50% cholesterol.

Results

Conformation of the Phosphatidylcholine and Phosphatidylethanolamine Head-Groups. An essential assumption of the analysis to follow is that the conformation of the phosphate moiety of the phosphocholine and phosphoethanolamine dipole remains essentially constant in bilayers of pure lipids or lipid mixtures. We provide two pieces of evidence to support this assumption.

Figure 1 (top panel) shows a summary of the quadrupole splittings vs temperature for *E. coli* PE specifically labeled at the 3,3-position of the glycerol backbone, i.e., the CH_2 segment attached directly to the phosphate moiety. The data were presented in a more limited form by Ghosh and Seelig (1982) and have now been extended to low temperatures. In all cases, the spectra were typical of an oriented fluid bilayer with well-resolved 0° edges of the so-called "powder pattern" (Seelig, 1977) and show the 3,3-^{2}H segment of the glycerol backbone to be undergoing rapid axial motion in the plane of the bilayer. $\Delta\nu_Q$ was determined by measuring the separation of the two most intense peaks of the powder pattern and was related to the deuterium order parameter, S_{CD}, according to

$$\Delta\nu_Q = (3/4)(e^2qQ/h)S_{CD} \quad (1)$$

where e^2qQ/h is the static quadrupole coupling constant (−170 kHz for a methylene group) and S_{CD} describes the angular fluctuations ($\bar{\theta}$) of the C–D bond around the bilayer normal:

$$S_{CD} = \overline{(3\cos^2\theta - 1)}/2 \quad (2)$$

Most noticeably, the sharp well-defined powder spectrum observed for bilayers of PE/cholesterol (1/1) remains easily observable for temperatures well below the T_c of the pure lipid.

In the presence of cholesterol, a small change in $\Delta\nu_Q$ is observed which is reflected also at the 2- and 1-positions of the glycerol backbone and is thought to represent a slightly altered conformation (Ghosh & Seelig, 1981). ^{2}H NMR spectra of *E. coli* [3,3-*glycerol*-^{2}H]-PE/POPC (1/1) mixtures show only a very small change in $\Delta\nu_Q$ at this position. This shows that the conformation of this segment in PE in the presence of equimolar amounts of POPC remains almost identical with that of the pure lipid. Interestingly, the presence of POPC (T_c = −5 °C) also allows the observation of narrow ^{2}H NMR spectra to well below the T_c for *E. coli* PE, presumably by allowing axial averaging to continue to these temperatures. Finally, the ^{2}H T_1 relaxation time measurements of bilayers of *E. coli* PE in the presence and absence of equimolar cholesterol are shown in Figure 1 (bottom panel). We discuss these data in detail below, but we note in passing that cholesterol has apparently no influence upon the motional averaging of the 3,3-^{2}H segment. Since the ^{2}H T_1 relaxation time is partly dependent upon the *order* of the segment (Brown et al., 1979), these data are further evidence that cholesterol does not influence the *structure* of this segment. Identical ^{2}H T_1 relaxation times were also observed for *E. coli* [3,3-*glycerol*-^{2}H]-PE/POPC (1/1).

We have also examined the order of the phosphocholine dipole using ^{14}N NMR. ^{14}N nuclei also possess a large quadrupole moment, and thus powder samples give rise to axially asymmetric powder spectra when measured with the quadrupole echo sequence. Recently, it has been shown (Siminovitch et al., 1980; Rothgeb & Oldfield, 1981) that the electric quadrupole tensor of the ^{14}N nucleus is averaged both by rapid rotation of the methyl groups of the choline moiety of PC and also by rapid axial diffusion of the phospholipid head-group about the bilayer normal, yielding powder spectra which are easily obtainable using standard instrumentation. For PE, only the second of the above averaging mechanisms exists, which is not sufficient to average the quadrupole tensor significantly, and to date, no broad-band ^{14}N NMR spectra have been reported for this phospholipid (Rothgeb & Oldfield, 1981; this study, data not shown).

Thus, in a mixture of PC and PE, only the PC component is detectable. Unfortunately, ^{14}N NMR powder spectra of phospholipids are still difficult to analyze; the value of the quadrupole coupling constant is not known with certainty although a value of 13 kHz has been proposed (Rothgeb & Oldfield, 1981); the principle axis of the ^{14}N electric tensor has not been ascertained, although it has been suggested to be the C_β–N bond. Nevertheless, ^{14}N NMR is extremely useful for comparative studies of the order of the choline moiety in bilayers of PC and PC/PE mixtures. Figure 2 shows ^{14}N NMR spectra of three systems used in this study. At 25 °C, the ^{14}N NMR spectra of bilayers of POPC (Figure 2, top) are sharp and well-defined with a quadrupole splitting of 11 kHz, essentially identical with those obtained for DPPC, DMPC, and DOPC in the liquid-crystalline phase (Rothgeb & Oldfield, 1981). In the presence of equimolar POPE (Figure 2, middle), $\Delta\nu_Q$ decreases by about 10%, which is approximately equivalent to that produced in bilayers of DPPC, DOPC, and DMPC, respectively, in the presence of equimolar cholesterol in the liquid-crystalline phase. The latter effect is thought to arise from the "spacing" effect of cholesterol, upon the lipid head-groups, allowing the choline moiety to move slightly out of the plane of the membrane. Substitution of *E. coli* PE for POPE (Figure 2, bottom) leaves $\Delta\nu_Q$ unchanged, but a small shoulder is observed between the two central peaks. It has been shown that ^{14}N NMR spectra of the gel phase have the same quadrupole splitting as those of the liquid-crystalline phase but are dramatically broadened (Siminovitch et al., 1980; Rothgeb & Oldfield, 1981). This suggests that a small fraction of the POPC is in the gel phase at this temperature, presumably due to an interaction with *E. coli* PE (T_c = 25 °C). In summary, the ^{14}N NMR data show that the order of the phosphocholine moiety remains almost unchanged in the presence of PE and does not vary with the nature of the fatty acyl chains.

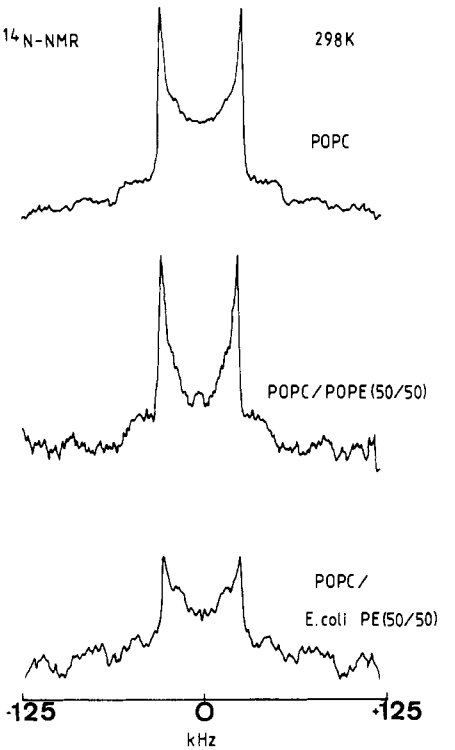

FIGURE 2: ^{14}N NMR spectra at 21.68 MHz for bilayers of POPC, POPC/POPE (1/1), and POPC/*E. coli* PE (1/1), respectively, dispersed in buffer at 25 °C. Spectra were recorded using the quadrupole echo sequence and with quadrature phase detection. Two hundred milligrams of lipid was used to obtain spectra of POPC alone and 150 mg total lipid for the other spectra.

^{31}P NMR Spectra of Lipid Mixtures. Figure 3 shows two sets of representative ^{31}P NMR "powder" spectra obtained for bilayers of *E. coli* PE and a mixture (1/1) of *E. coli* PE and POPC. The chemical shielding anisotropy, $\Delta\sigma$, obtained from the distance between the peak and shoulder of the spectrum is −41 ppm above 30 °C, characteristic of PE in the liquid-crystalline phase [see Seelig (1978)]. As the temperature is decreased, the rate of axial motion decreases, and $\Delta\sigma$ is no longer rapidly averaged ($\gamma_P H_0 \tau_c \ll 1$), leading to a gradual broadening of the spectrum. The gel phase spectrum ($\Delta\sigma$ = −75 ppm) is obtained at approximately 0 °C. The phase transition observed is consistent with that observed by differential scanning calorimetry measurements (F. Borle and J. Seelig, personal communication). Figure 3 shows ^{31}P NMR spectra obtained from bilayers containing a mixture of *E. coli* PE and POPC (1/1) under the same conditions as those used for *E. coli* PE alone. At higher temperatures, where each of the pure lipid bilayers adopts the liquid-crystalline phase, the observed spectra correspond simply to the superposition of the individual components with chemical shielding anisotropies of approximately −41 and −47 ppm, corresponding to *E. coli*

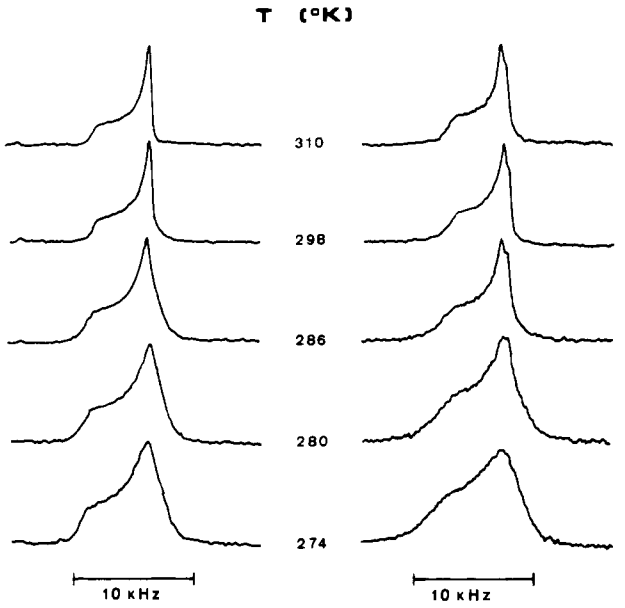

FIGURE 3: ^{31}P NMR spectra at 121.4 MHz for liposomes of *E. coli* PE (left) and POPC *E. coli* PE (1/1) (right). The pure dried lipid was dispersed in buffer directly, whereas the lipid mixture was dissolved first in chloroform, dried under high vacuum for 12 h, and then dispersed as above. All spectra were taken using high-power gated proton decoupling and a spectral width of 31.25 kHz. For the spectra obtained from the lipid mixtures, the inner and outer field peaks represent the contributions from PE and PC, respectively.

PE and POPC, respectively. Indeed, two distinct components are easily observable. As the temperature is decreased, the spectra broaden, but two components at the low-field edge are always observable until the lowest temperatures are (>2 °C) are reached. In all of the systems studied here, we have observed no evidence of phase separation (by the criterion of spectral superposition or inhomogeneity of the ^{31}P T_1 relaxation time; see below). This is probably because changes of temperature were made very slowly, thus allowing the bilayer components to reach an equilibrium state.

Figure 4 shows a summary of the apparent $\Delta\sigma$ obtained from various lipid mixtures. This apparent $\Delta\sigma$ is only approximate and corresponds most closely to the head-group of PC since the measurement is taken from the outermost edges of the powder spectrum. In Figure 4 (top), mixtures with differing head-group ratios but with identical fatty acyl chains were examined. Pure POPE was not included in this study as it preferentially assumes the hexagonal H_{II} phase at temperatures above the phase transition. Instead, the minimum amount of POPC required (20%) to maintain the bilayer phase at all temperatures was included. In fact, the onset temperature of the observed phase transition (approximately 22 °C) of this mixture corresponds closely to that observed for bilayers of pure POPE (T_c = 16 °C) using DSC.

The apparent $\Delta\sigma$ of an equimolar mixture of POPC/POPE corresponds closely to that of POPC alone, and the spectra (not shown) appeared at all temperatures to correspond to those shown in Figure 3 (right) at 25 and 37 °C.

Figure 4 (bottom) summarizes the data shown in Figure 3 and shows PC/PE mixtures where the fatty acyl chain complement is inhomogeneous. Note that although the phase transition for *E. coli* PE is much broader than that for POPE alone, it occurs over approximately the same range. In contrast to bilayers of equimolar POPC/POPE, however, the $\Delta\sigma$ vs T profile does not follow that of pure POPC closely below 20 °C but rises to meet that of pure *E. coli* PE at 10 °C. The observed phase transition of bilayers of equimolar *E. coli* PE/POPC does not appear to be the average of that of the

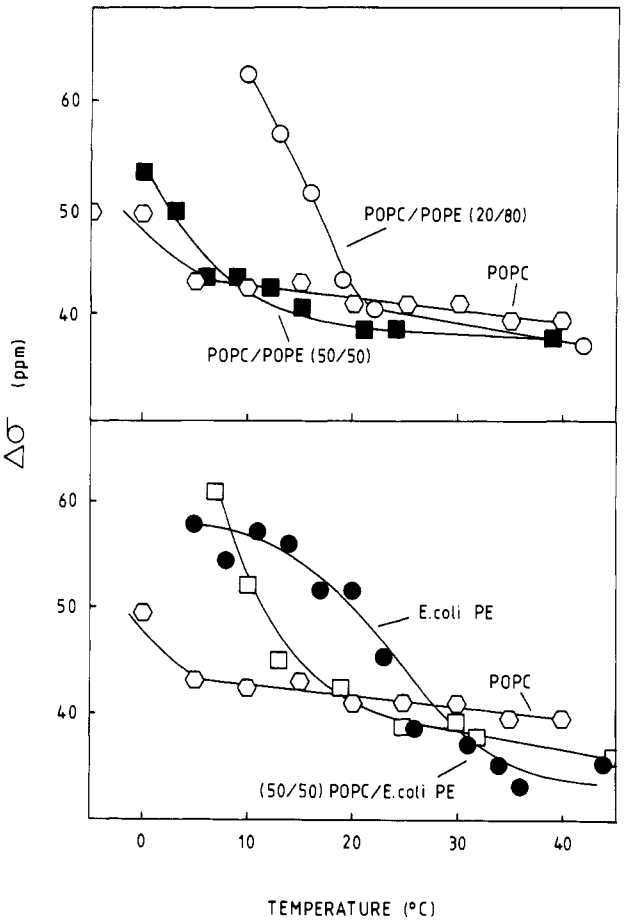

FIGURE 4: Variation of the apparent chemical shielding anisotropy, $\Delta\sigma$, with temperature at 121.4 MHz for liposomes of varying lipid composition (symbols are indicated in the figure). (top) Variation of the head-group composition for a homogeneous fatty acyl chain complement; (bottom) variation of the head-group composition for a heterogeneous fatty acyl chain complement.

two pure lipids, but rather a shift of the POPC profile toward higher temperatures. Once again, no evidence of phase separation was observed for the ^{31}P NMR spectra (Figure 3, right) or for the ^{31}P T_1 relaxation times (see below). That the apparent $\Delta\sigma$ vs T profile corresponds to the physical behavior of both components of the *E. coli* PE/POPC (1/1) bilayer is corroborated by the observation that liquid-crystalline ^2H NMR spectra can be obtained by using the 3,3-^2H-labeled *E. coli* PE/POPC mixture to temperatures below those for the pure 3,3-^2H-labeled *E. coli* PE alone (Figure 1). The effect of cholesterol upon the quadrupole splitting of 3,3-^2H-labeled *E. coli* PE corresponds to data already published by several groups (Brown & Seelig, 1978; Rice et al., 1979; Blume et al., 1982a,b).

^{31}P T_1 Relaxation Studies. Figure 5 shows a typical ^{31}P T_1 relaxation profile of a bilayer of *E. coli* PE/POPC (1/1) in the liquid-crystalline phase. Most significantly, the ^{31}P NMR spectra relax homogeneously and can be fitted well by a single correlation time. This result was found to be true for all lipid mixtures examined at all temperatures presented in this study. However, great care was taken to ensure that the lipid mixtures reached thermal equilibrium (usually 20–30 min) and that the probe head was properly tuned before each measurement. For mixtures of lipids, especially those from natural sources such as *E. coli* PE [though we have obtained similar results from sarcoplasmic reticulum and mitochondria (unpublished data)], the first parameter was of critical importance. The variation of the ^{31}P T_1 relaxation time vs temperature for the lipid mixture studied here is shown in

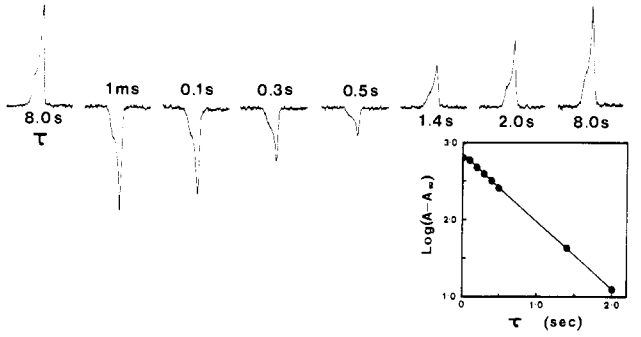

FIGURE 5: Measurement of the ^{31}P T_1 relaxation time at 121.4 MHz for liposomes containing POPC/*E. coli* PE in buffer using the inversion recovery technique. Measurements were made without proton decoupling. Insert: Semilogarithmic representation for the ^{31}P T_1 data vs delay time (t).

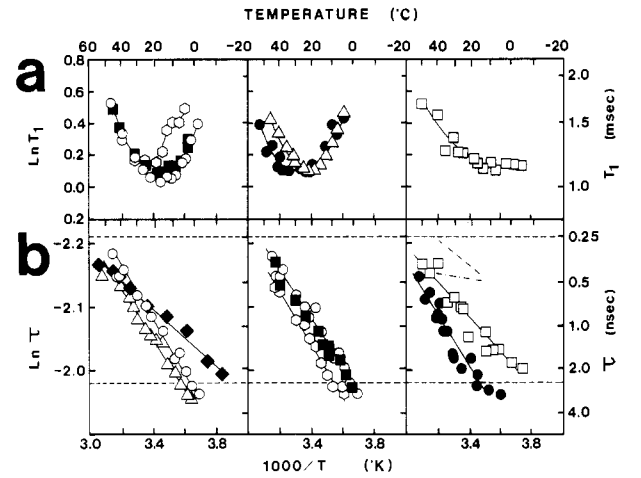

FIGURE 6: (a) Variation of the ^{31}P T_1 relaxation time at 121.4 MHz with temperature for liposomes of varying lipid composition. The phospholipid composition is given by the following symbols: DOPC (♦); POPC (○); POPC/POPE (1/1) (■); POPC/POPE (1/4) (○); POPC/*E. coli* PE (1/1) (△); *E. coli* PE (●); *E. coli* PE/cholesterol (1/1) (□). The kink observed for the ^{31}P T_1 vs. T profile of POPC/POPE (1/4) corresponds to the onset of the phase transition. (b) Arrhenius representation of the data from (a) deconvoluted using eq 5 (see text) to yield apparent correlation times for various temperatures. The solid lines were calculated by linear regression. The dash–dot lines represent the correlation time data obtained from the ^2H T_1 relaxation time measurements of liposomes of 3,3-^2H-labeled (upper) and 2-^2H-labeled *E. coli* PE (lower), respectively (Ghosh & Seelig, 1982), and the horizontal dashed lines represent the correlation times ($\tau_\parallel$, upper; $\tau_\perp$, lower) obtained for spin-labeled cholesterol in bilayers of DPPC at 40 °C using ESR spectral simulation techniques (Schindler & Seelig, 1974).

Figure 6a. The most striking feature of these data is that a minimum is observed. At the position of the minimum, the correlation time passes from the high ($\omega_p\tau_c \ll 1$) to the low ($\omega_p\tau_c \gg 1$) correlation regime. We note that the *position* ($\tau_c = 0.935\omega_p$) and the *amplitude* of the minimum vary with the lipid system under investigation. For instance, the incorporation of equimolar amounts of POPE into a bilayer of POPC has almost no effect upon the relaxation behavior observed with the exception of a small change in amplitude. In contrast, the amplitude of the ^{31}P T_1 vs T profile of the POPC/POPE (1/4) mixture is dramatically shifted. The small discontinuity observed on the low correlation side of the latter curve is more probably due to the onset of the phase transition at this temperature. Introduction of fatty acyl chain heterogeneity into an equimolar PC/PE mixture [in *E. coli* PE/POPC (1/1)] has no effect upon the relaxation behavior as compared to that with the homogeneous distribution of chains [i.e., POPE/POPC (1/1)]. The temperature dependence of the ^{31}P T_1 relaxation time observed for bilayers of *E. coli* PE is somewhat broader although the amplitude of the minimum remains unchanged. Addition of equimolar amounts of cholesterol to bilayers of *E. coli* PE causes a dramatic shift of the minimum to lower temperatures although the amplitude remains identical. Finally, we note the large degree of scattering of ^{31}P T_1 data points observed for bilayers of *E. coli* PE or *E. coli* PE/cholesterol (1/1) as compared to those of pure lipids. Incorporation of equimolar POPC in bilayers of *E. coli* PE had a dramatic effect in reducing this scattering.

Discussion

The main purpose of this work is to extend the analysis of Seelig et al. (1981) and show that studies of the ^{31}P T_1 relaxation times of phospholipid bilayers can indeed lead to a meaningful understanding of the principal motional events involved at the level of the phospholipid head-group.

Head-Group Conformation in Pure Lipids and Mixtures of Phospholipids. It is now well accepted that dipolar and quadrupolar relaxation equations contain a term determined by the *order* of the motional moiety involved (Brown et al., 1979; Brown, 1984a). In this study, we have used ^2H, ^{14}N, and ^{31}P NMR to show that the *order* of the head-group remains essentially constant under the conditions studied.

For instance, ^2H NMR spectra of the 3,3-^2H position of the glycerol backbone of *E. coli* PE show that the order of this CD_2 segment, as measured directly by the quadrupole splitting $\Delta\nu_Q$, remains essentially unchanged in the presence of equimolar amounts of POPC or cholesterol. In addition, the ^2H NMR spectra observed at all temperatures and for all lipid mixtures used in this study are characteristic of a CD_2 segment in the liquid-crystalline phase, undergoing rapid axial rotation ($>10^4 s^{-1}$) sufficient to average the quadrupole tensor.

In addition, it has been suggested previously that this segment is undergoing rapid trans–gauche isomerization [$\sim 10^{-10}$ s (Seelig & Gally, 1976; Brown et al., 1979)]. We note that this segment is attached directly to the phosphate group at the glycerol backbone, and thus their motion must be correlated in some way. Due to the similar values of S_{CD} and the ^2H T_1 relaxation times from a variety of PE's and PC's (Gally et al., 1977; Brown et al., 1979; Browning & Seelig, 1981; Browning, 1981a,b,c), we expect no large differences in the structure and motion of this segment for the different phospholipid classes studied here.

We have used ^{14}N NMR as a probe of head-group conformation in POPC/POPE and POPC/*E. coli* PE mixtures. The advantage of this technique is that the quadrupole tensor of the tertiary nitrogen of the PC head-group is sufficiently averaged by rotational diffusion to yield observable spin 1 powder spectra (Rothgeb & Oldfield, 1981). In all cases, "powder spectra" were obtained with sharply defined peaks, indicating that at the temperature studied the head-group of PC is in the liquid-crystalline phase. An unambiguous interpretation of the quadrupole splitting is difficult as the quadrupole coupling constant (e^2qQ/h) and the principal axis of the quadrupole tensor are not known, although the latter is likely to be the C_β–N axis (Rothgeb & Oldfield, 1981). However, it is safe to conclude from the sharpness and width of the ^{14}N NMR spectra that the quadrupole splitting observed may be taken as a measure of the angular fluctuations of the C_β–N axis with respect to the bilayer normal and that this is quite similar for bilayers of pure PC, mixtures of PC and PE (up to 80% PE; this study), and mixtures of PC and cholesterol in molar ratios of up to 50% (Rothgeb & Oldfield, 1981).

This conclusion is corroborated by examination of the ^2H NMR data published for pure and mixtures of PC and PE in

phospholipid bilayers. To compare these data we will use the concept of "reduced temperature" (T_{red}) introduced by Seelig and Browning (1981) [$T_{red} = (T - T_c)/T_c$] where T is the measured temperature and T_c is the phase transition temperature of the pure phospholipid component. In the liquid-crystalline phase at a reduced temperature of 0.428 (60 °C for DPPC; 90 °C for DPPE), the respective quadrupole splittings for C_α and C_β ^{2}H-labeled CD_2 segments of DPPC and DPPE are quite similar [$\Delta\nu_Q[C_\alpha(DPPC/DPPE)] = 6$ kHz/5–8 kHz; $\Delta\nu_Q[C_\beta(DPPC/DPPE)] = 4.2$ kHz/4 kHz) (Gally et al., 1975; Seelig & Gally, 1975; Seelig et al., 1977)]. This similarity is retained to the temperature of the phase transition. At the lower reduced temperature of 0.1, the quadrupole splitting for the $C_\beta D_2$ segment of DPPE (9.5 kHz) remains almost unchanged in the presence of up to 80% DPPC and is only reduced by 13% in the presence of 50% cholesterol (Seelig & Gally, 1976; Brown & Seelig, 1978; Blume et al., 1982a,b; Blume & Griffin, 1982). The latter change corresponds well to that observed for $\Delta\nu_Q$ for the choline nitrogen of DPPC in the presence of 50% cholesterol (Rothgeb & Oldfield, 1981).

Finally, we move to the analysis of the ^{31}P NMR powder spectra shown in Figure 2. As the observed chemical shielding anisotropy is determined by two order parameters (eq 3)

$$\Delta\sigma = (\sigma_{11} - \sigma_{22}) + S_{33}(\sigma_{33} - \sigma_{22}) \quad (3)$$

(where σ_{ii} are the principal elements of the chemical shielding tensor in the molecular frame and S_{ii} are order parameters) whereas only one measurement ($\Delta\sigma$) is available from a powder, an unambiguous structural determination of the data is not possible (Skarjune & Oldfield, 1976). Nevertheless, it has been demonstrated repeatedly that the chemical shielding anisotropy observed for a pure phospholipid bilayer in the liquid-crystalline phase is almost invariant with temperature and also of the nature of the fatty acyl chains. This was also observed in this study. In addition, due to the slightly different values of the principal components of the chemical shielding tensor, the $\Delta\sigma$ of PE (−41 ppm) is somewhat smaller than that of PC (−47 ppm) (Seelig & Gally, 1976; Seelig, 1977). For phospholipids in the liquid-crystalline phase containing both PC and PE head-groups, two components are indeed observed, with $\Delta\sigma$ corresponding to those for bilayers of the isolated components, respectively. Assuming that the observed $\Delta\sigma$ is determined principally by the small change in head-group conformation observed for the phospholipid crystal structure (Hitchcock et al., 1974; Pearson & Pascher, 1974), we conclude that the head-group structure is not significantly perturbed by mixing. This is corroborated by the data obtained for the 3,3-^{2}H segment of the glycerol backbone adjacent to the phosphate moiety of *E. coli* PE (Figure 1). In addition, Sixl and Watts (1982) have shown using ^{31}P NMR that the head-group conformations of both components of bilayers of DMPC/DMPE (1/1) are relatively unaffected by mixing.

The ^{31}P NMR spectra also yield useful information as to the mixing of the two phospholipid components. Figure 4 shows experiments where (a) the composition and stereochemistry of the fatty acyl chains are homogeneous for both phospholipids and (b) the composition of the fatty acyl chains is heterogeneous for one of the two phospholipids. As expected from the DSC data, the $\Delta\sigma$ of POPC remains characteristic of the liquid-crystalline phase until 5 °C whereupon it rises steeply as the gel phase is entered (Tamm & Seelig, 1983). Similarly, POPC/POPE (1/4) mixtures show a temperature variation of $\Delta\sigma$ closely resembling the thermal transition observed for pure POPE (+5 °C), with the onset of the gel phase commencing at 20 °C. However, mixtures containing 50% POPE do not show an averaging of these phase transitions but appear to be retarded with the observed onset of the phase transition closely following that of pure POPC. This strongly suggests that at equimolar ratios POPC and POPE are well mixed, with POPC "spacing out" the PE head-group, thus weakening the H-bonding interactions which lead to a higher phase transition temperature. Phase separation is not observed, neither in the ^{31}P or ^{2}H NMR spectra nor in the ^{31}P or ^{2}H T_1 relaxation times. This is probably because the fractional change in temperature between measurements was small, and sufficient time was allowed for a new equilibrium point to be reached. When the fatty acyl chain composition is heterogeneous (Figure 4, bottom), this delaying effect of POPC upon the phase transition is not so marked. Nevertheless, as judged from the ^{31}P NMR spectra (Figure 3) and the ^{31}P T_1 and ^{2}H T_1 relaxation behavior, the two lipid components are "well-mixed" within the bilayer at all temperatures examined here.

Analysis of the ^{31}P T_1 Relaxation Times for Bilayers of Phospholipids and Phospholipid Mixtures. (A) General Aspects. One of the fundamental observations of this study is that all of the ^{31}P NMR spectra presented here relax homogeneously at all temperatures studied; i.e., the ^{31}P T_1 relaxation time is essentially identical for the angular distribution of phosphate groups about the magnetic field. This observation suggests a continuous diffusion relaxation model, in the simplest case a rapidly rotating ellipsoid experiencing an ordering potential. Such motion may be described by two correlation times, $\tau_\perp$ and $\tau_\parallel$, which describe the motion of the body parallel and perpendicular to the membrane normal. Should these correlation times be separated by a factor of 10, then only one may dominate under most conditions, and the relaxation behavior may be described by a single correlation time τ_c. We have chosen to extract τ_c phenomenologically by modifying the model of Doddrell et al. (1972) which is based on the theory of Woessner (1962):

$$1/T_1^{exp} = 1/T_1^{DD} + 1/T_1^{CSA} \quad (4)$$

$$1/T_1^{exp} = \alpha\sum J(\omega_i) + \beta(H_0)J(\omega_i) \quad (5)$$

(see the Appendix). In doing so, we assume an "effective proton" which probably corresponds to a cluster of protons situated in the plane of the head-groups in the bilayer. For instance, Yeagle et al. (1977) suggested that PC bilayers these protons correspond to the choline methyl groups since (i) deuteriation of the choline methyl protons causes a shift of the NOE to the methylene groups and (ii) replacement of bulk water by D_2O has little effect upon ^{31}P T_1 relaxation, and cholesterol, which acts as a head-group spacer, also induces a shift of the NOE to the methylene groups. All ^{31}P T_1 relaxation measurements in this study have been performed on pelleted liposomes so that residue tumbling can be excluded as a relaxation mechanism.

(B) What Are the Physical Mechanisms Causing ^{31}P T_1 Relaxation in Phospholipid Bilayers? We begin by turning to the analysis of the ^{2}H T_1 relaxation behavior of [3-^{2}H]-cholesterol in bilayers of *E. coli* PE (Ghosh & Seelig, 1982; this study). The ^{2}H T_1 relaxation times of the 3-^{2}H-labeled position in bilayers of *E. coli* PE are some of the shortest observed so far, and the temperature dependence of the relaxation is only weak. As the ring system of cholesterol experiences no flexing motions, Brainard and Szabo (1981) were able to resolve two correlation times using ^{13}C NMR to describe the motion of cholesterol in a phospholipid bilayer: $\tau_\perp$ (~2.5 ns) and $\tau_\parallel$ (~0.25 ns). The condition $\tau_\perp/\tau_\parallel > 10$ is satisfied, and Ghosh and Seelig (1982) were able to assign the observed τ_c for the [3-^{2}H]cholesterol of approximately 0.36

Table I: Activation Energies for Lipid Bilayers of Varying Composition[a]

lipid	E_a (kJ mol^{-1})	K (=[F$_0$]^2)	T_1 (s)	τ_c (ns) at 20 °C
DOPC	18.6	1.7×10^8	1.05	0.6
POPC	31.4	1.46×10^8	1.2	0.62
POPC/POPE (1/1)	30.5	1.29×10^8	1.23	0.675
POPC/POPE (1/4)	34.5	1.14×10^8	1.18	0.92
POPC/*E. coli* PE (1/1)	32.9	1.2×10^8	1.16	0.91
E. coli PE	32.4	1.29×10^8	1.16	1.65
E. coli PE/chol (1/1)	22.6	1.22×10^8	1.29	0.65

[a] K and τ_c were determined by the procedure given in the Appendix using eq A2 as described. The activation energy (E_a) was determined from the slope of the ln τ_c vs T data.

ns at 45 °C to that of $\tau_\perp$. Surprisingly, the variation of ^{2}H T_1 relaxation with temperature for the 2-^{2}H-labeled segment of the glycerol backbone of *E. coli* PE may be superimposed almost exactly upon that of [3-^{2}H]cholesterol, and above the phase transition is *identical* in the absence or presence of equimolar cholesterol. As the glycerol backbone is a rigid structure, it would suggest that the τ_c observed for the [2-^{2}H]glycerol/*E. coli* PE is determined principally by overall phospholipid rotational diffusion. The ^{2}H T_1 temperature profile for the 2-^{2}H-labeled *E. coli* PE in bilayers is shown as an intermittent line intersecting the ^{31}P T_1 data at 40 °C and has an activation energy (E_a) of 15.6 kJ mol^{-1} (cf. Table I). The activation energy and correlation time remain almost unchanged in the presence of cholesterol, in stark contrast to the well-known effect of cholesterol upon lateral diffusion which may be decreased by an order of magnitude (Owicki & McConnell, 1980). This result is in agreement with recent dynamic fluorescence anisotropy measurements of diphenylhexatriene in liposomes of POPC and POPC/cholesterol mixtures (Kuwato et al., 1978) and of ^{2}H NMR data of α- and β-cholesterol embedded in DMPC (Dufourc et al., 1984).

The dashed lines in Figure 6b show the correlation times $\tau_\perp$ and $\tau_\parallel$ (corresponding to rotational and lateral diffusion) of a cholesterol molecule in phospholipid bilayers [see Ghosh and Seelig (1982)]. At 45 °C, the ^{31}P T_1 derived correlation times for bilayers of *E. coli* PE and *E. coli* PE/cholesterol correspond well to those for overall phospholipid rotation. However, as the temperature is decreased, the ^{31}P correlation times lengthen dramatically and deviate significantly from those of overall phospholipid rotation (E_a = 32 and 22 kJ mol^{-1} for bilayers of *E. coli* PE). We note that the activation energy for lateral diffusion in bilayers of POPC has been estimated to be approximately 30 kJ mol^{-1}, and so the lower dashed line will remain similarly distanced from the ^{31}P T_1 correlation times at all temperatures.

On the basis of the above conclusions, we propose the following: (i) Head-group motion in bilayers of phospholipid mixtures can be described by a single set of physical parameters. This suggests that interactions between head-groups lead to an averaging of the rates of rotational diffusion of these moieties. (ii) The correlation time of the phosphate moiety is determined principally by its rotational diffusion in the plane of the membrane.

This model can be used to describe the experimental data in Figure 6. Thus, the inclusion of a single double bond in the *sn*-2 fatty acyl chain of PC has a dramatic effect upon the activation energy, increasing it from 18.7 to 31.41 kJ mol^{-1} (see Table I). We interpret this as arising from the increased *spacing* between phospholipid head-groups due to increased fluctuations of the hydrocarbon chain. Thus, DOPC head-groups are less tightly packed and can rotate more rapidly than those of POPC. Replacing 50% of the head-groups by PE but keeping the hydrocarbon chain composition constant appears to have no effect upon head-group rotation (E_a = 30.5 kJ mol^{-1}) probably due to good mixing of the phospholipids; i.e., PE is unable to establish a long-range hydrogen-bonded network at these temperatures due to the perturbation by PC. The introduction of a heterogeneous mixture of fatty acyl chains into the bilayer [PC/*E. coli* PE (1/1)] also leaves head-group rotation unaffected (E_a = 32.9 kJ mol^{-1}). In fact, the fatty acyl composition of naturally occurring *E. coli* PE [see Lugtenberg and Peters (1976)] resembles that of POPE quite closely. Bilayers composed principally of POPE [POPC/POPE (1/1)] in the liquid-crystalline phase show a somewhat slower rate of head-group rotation with a slightly larger activation energy (E_a = 34.5 kJ mol^{-1}). This slowing effect of the hydrogen-bonding network is even more exaggerated in bilayers of *E. coli* PE alone, although the activation energy (E_a = 32.4 kJ mol^{-1}) is identical with that of mixtures of POPC/*E. coli* PE (1/1). It should be noted that bilayers of *E. coli* PE experience a rather broad phase transition (5–30 °C) (Figure 4) so that many of the ^{31}P T_1 relaxation measurements were performed in this range. However, extraction of correlation times from these data using the simple model described still yields linear Arrhenius plots except at temperatures below 10 °C where a large fraction of the population is in the gel phase. Blume and Griffin (1982) have shown for bilayers of DPPE that rapid exchange on the ^{2}H NMR time scale occurs between coexisting gel and liquid-crystalline phases at the phase transition temperature. For a naturally occurring phospholipid where the phase transition is broad, the cooperative unit (Mabrey & Sturtevant, 1976) is probably small, and rapid exchange occurs for a large fraction of the lipid molecules for most of the phase transition range. Thus, rapid rotational motion is still allowed until almost the end point of the phase transition, and the continuous diffusion model is applicable until the gel phase is reached. At 15 °C, where a large fraction of the *E. coli* PE phospholipids are in the gel phase, an apparent discontinuity occurs in the Arrhenius representation.

The presence of equimolar cholesterol in bilayers of *E. coli* PE abolishes the phase transition, possibly by allowing rotational diffusion of the PE molecules to continue below temperatures at which they would condense in the absence of cholesterol. This is also observed using the chemical shielding anisotropy of the ^{31}P NMR data (not shown) which does not broaden beyond the $\Delta\sigma$ observed for the liquid-crystalline phase until approximately −20 °C. The effect of cholesterol upon the correlation times of the PE head-group is dramatic, however, causing a reduction of τ_c by a factor of 2 and decreasing the activation energy (E_a = 22.6 kJ mol^{-1}) to almost that observed for pure DOPC. From the physical model presented here, cholesterol might increase the spacing between head-groups, thus preventing the formation of a long-range hydrogen-bonding network between neighboring PE molecules, weakening the electrostatic and steric restraints, and allowing head-group rotation to occur more freely in the pure lipid state at temperatures below the onset of the phase transition. At 50 °C, where *E. coli* PE is in the liquid-crystalline phase and large fluctuations of the fatty acyl chains occur, leading to increased spacing of the phospholipid molecules, the calculated correlation times for bilayers of pure PE and PE/cholesterol appear to coincide.

To summarize, the analysis of the ^{31}P T_1 relaxation times for the head-groups of phospholipids in the liquid-crystalline phase is dependent upon the observation of a T_1 *minimum*, which in turn allows the definition of the spectral density

region. Seelig et al. (1981) suggested that the observation of a T_1 minimum for phospholipid bilayers depends on the following fortunate combination of conditions: (a) relatively high field strength ($\omega_p = 7.6 \times 10^8$ rad s^{-1}); (b) a low phase transition temperature allowing sufficient slowing of the motion to occur at lower temperatures without the formation of the gel phase; (c) motion of the bulky phosphate group is slowed additionally by inter-head-group interactions.

We have extended the model of Seelig et al. (1981) with the following conclusions: (i) In the region of the ^{31}P T_1 minimum, the motion of the phosphate moiety of the phospholipids in the liquid-crystalline phase may be described by a single correlation time. (ii) The correlation time, τ_c, derived from the ^{31}P T_1 data from PE and PC pure lipids and mixtures may be assigned to the correlation time of head-group rotational diffusion in the plane of the membrane. This conclusion may not be strictly true; nevertheless, it yields an interpretation of the data which appears to be consistent with that obtained using other techniques. (iii) Head-group rotation is not obligatorily linked to overall phospholipid rotation (as described by the motion of the glycerol backbone). Indeed, cholesterol appears to cause an increase in the motion of PE head-groups without affecting the motion of the glycerol backbone.

Acknowledgments

I thank Professor Joachim Seelig for his support and encouragement of this work and P. Ganz for the synthesis of the deuteriated phospholipids.

Appendix

Assumptions. (1) The form of the spectral density function is determined principally by a single correlation time. This assumption holds if a single kind of motion under a uniform interaction dominates the longitudinal relaxation terms. (2) The ^{31}P T_1 relaxation time is determined by only two principal contributions: the dipole–dipole interaction and the chemical shielding anisotropy (Seelig et al., 1981). (3) In mixtures of phospholipids, the spectra relax homogeneously according to a single exponential. This is, in fact, an experimental observation and should be tested for each case individually.

Following Seelig et al. (1981), ^{31}P T_1 relaxation in phospholipids may be described by the equation:

$$1/T_1 = 1/T_1^{DD} + 1/T_1^{CSA} \tag{A1}$$

$$1/T_1 = K[J(\omega_H - \omega_p) + 3J(\omega_p) + 6J(\omega_H + \omega_p)] + \alpha(\Delta\sigma)^2\omega_p^2 J(\omega_p) \tag{A2}$$

where the spectral density functions $[J(\omega_i)]$ are

$$J(\omega_i) = \tau_c/(1 + \omega_i^2\tau_c^2) \tag{A3}$$

and K and α are empirical constants to be determined. We assume further that the spectral density functions are correct as written even though eq A2 is only strictly valid for isotropic motion and some cases involving restricted motion. For the latter cases, the coupling constants must be scaled by order parameters [see Brainard and Szabo (1981)] which is implicit in the empirical determination of K and α.

At low field (2.25 T)

$$1/T_1^{obsd} = Kf(\tau_c) \tag{A4}$$

and at high field (7.05 T)

$$1/T_1^{obsd} = Kf(\tau_c) + \alpha(\Delta\sigma)^2\omega_p^2 g(\tau_c) \tag{A5}$$

If the position of the minimum $\tau_c = \tau_c^*$ is known, then at low field $\tau_c \sim 0.8\ (1/\omega_p)$, and the value of K may be determined and hence the variation of τ_c vs T. Conversely, having thus determined K, the high-field data can be used to determine α, once again at $\tau_c = \tau_c^*$ (in practice, α is determined for several values of known τ_c). In fact, when two field strengths are used, the value of K has been determined for only a single lipid (DOPC) (Seelig et al., 1981) and was found to be 1.65×10^8 s^{-2}. The corresponding linear variation of ln τ_c vs T was determined by using this value only from the low-field data, and the activation energy was found to be 4.08 kcal mol^{-1}. In the present study, we have determined α to be 1.7×10^8 s^{-2} and derived ln τ_c vs T solely from the high-field data by substituting K and α into eq A3 and A4 and subsequently, by computer simulation, reading off the values of τ_c for experimental values of T_1. This procedure yielded a curve of ln τ_c vs T almost directly superimposable upon that given by Seelig et al. (1981) with an activation energy of 4.46 kcal mol^{-1}.

Unfortunately, extensive measurements at two field strengths are often prohibitively time consuming, as evidenced by the scarcity of such data in the literature, and it is thought that ^{31}P T_1 vs T data cannot be analyzed in terms of τ_c from measurements at a single field strength. However, we show now that the parameters obtained from DOPC together with the observation of a minimum in the ^{31}P T_1 data are sufficient to analyze the corresponding relaxation behavior from bilayers of unknown lipids in the liquid-crystalline phase using only data obtained at high field.

To proceed further, we note two facts. First, if the measurements are restricted to the liquid-crystalline phase, then the variation in $\Delta\sigma$ (see Figure 4) is small (approximately 10–20%), and thus the term $(\Delta\sigma\omega_p)^2$ in eq A3 remains essentially constant at all temperatures. Second, the numerical value of α depends on the order and motional average of the phosphate segment. As these parameters are essentially identical and constant for the phosphocholine and phosphoethanolamine dipoles in the liquid-crystalline phase, α may be assumed constant and equal to the numerical value derived for bilayers of DOPC. Thus, the large variations in amplitude (i.e., the T_1 abscissa) of the ^{31}P T_1 data are produced by a *variation in the dipolar interaction term, K.* The final step is to adjust the value of K for eq A2, keeping α constant, so that the position of the theoretical T_1 minimum corresponds to the experimental one. The correlation time–temperature dependence may then be read off directly. The applications and limitations of this analysis are discussed in the main text.

Registry No. DOPC, 4235-95-4; POPC, 26853-31-6; POPE, 26662-94-2; cholesterol, 57-88-5.

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