Phosphorus-31 two-dimensional solid-state exchange NMR

Application to model membrane and biological systems

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ABSTRACT Two-dimensional solid-state ^{31}P NMR has been used to investigate the orientational exchange of phospholipids in gel and liquid-crystalline aqueous multilamellar dispersions and oriented multibilayers, and in biological membranes. In liquid-crystalline L_α multilamellar dispersions, orientational exchange originates from the lateral diffusion of phospholipid molecules over the curved surface of the liposomes and is manifest by an increase in off-diagonal intensity, which correlates the 90 and 0° orientations of the membrane normal with respect to the magnetic field when the system is fully exchanged. Spectral simulations of the time evolution of exchange allowed determination of the correlation times τ_d for lateral diffusion. For DMPC and DPPC at comparable reduced temperatures, τ_d values of 44 and 8 ms were obtained, respectively. The nature and rate of exchange observed for POPE at 30°C is similar to that of DMPC at the same temperature. The measured correlation times are consistent with diffusion rates obtained by FRAP for liposomes with radii in the 1 μm range. In the gel phase of DPPC (30°C), little orientational exchange is observed at mixing times up to 200 ms, demonstrating that the lateral diffusion is very slow. The correlation time for orientational exchange obtained from spectral simulations was ~900 ms; thus, exchange in the gel state is at least two orders of magnitude slower than in the liquid-crystalline state. In the $P_{\beta'}$ (ripple) phase, at temperatures between 34 and 39°C, significant exchange is observed for mixing times between 50 and 200 ms. Exchange is also observed in oriented samples of DPPC in the $P_{\beta'}$ phase for mixing times of 50 ms, but not for oriented liquid-crystalline samples for mixing times up to 100 ms. The exchange observed in the ripple phase could originate from rapid lateral diffusion of "fast" diffusing phospholipid within defect structures, and/or from "slow" lateral diffusion of ordered phospholipid over the ripples. 2D experiments were also performed on pig erythrocyte ghosts and on intact pig spinal cord. Significant orientational exchange was observed with the erythrocyte ghosts at a mixing time of 200 ms, but almost no exchange was observed with the spinal cord at the same mixing time. Spectral simulations suggest τ_d values of ~400 ms and 1.3 s for the erythrocyte ghosts and spinal cord at 30°C. The results demonstrate that exchange in the biological membranes is significantly slower than in the model membrane systems, which suggests that the cell surfaces are relatively "smooth," i.e., any local surface perturbations are either present in small number or have little effect on the mean orientation of the phospholipids with respect to the membrane normal.

INTRODUCTION

The application of solid-state NMR techniques to the study of model and biological membranes has yielded a wealth of information relating to molecular orientations, as well as types and rates of motions of lipid molecules. ^{31}P NMR has proven particularly useful in characterizing the orientational and dynamic properties of phospholipid headgroups (Seelig, 1978; Seelig et al., 1981; Campbell et al., 1979; Milburn and Jeffrey, 1987, 1989) and has been extensively applied in the study of lipid polymorphism (Cullis and de Kruijff, 1979). These applications, utilizing a combination of lineshape and spin-lattice relaxation studies, allow molecular motions to be probed in the frequency range of 10^3–10^{10} Hz.

The above approaches are not sensitive to slower molecular motions ($<10^3$ Hz). These are, however, accessible to study by two-dimensional NMR, which extends the frequency range of study down to 10^0 Hz. The majority of 2D-exchange experiments on solids have been performed on samples which yield high-resolution spectra (e.g., by using single crystals or CP/MAS techniques). Only recently has two-dimensional exchange NMR been applied to static powders; these studies measured chemical exchange and spin diffusion via deuteron (Schmidt et al., 1986, 1988) and ^{13}C (Edzes and Bernards, 1984; Hagemeyer et al., 1989) 2D NMR, respectively. To date there has been only one application of (deuteron) 2D-exchange NMR to membrane systems, in which a slow whole molecule motion was demonstrated in the gel phase of hydrated multibilayers of the glycolipid 1,2-di-*O*-tetradecyl-3-*O*-(β-D-glucopyranosyl)-*sn*-glycerol (Auger and Jarrell, 1990). The major limitations in using deuterium in 2D studies are the need for chemical labeling, the low sensitivity, and the generally short relaxation times, which limit the time scale

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over which exchange can be observed. The only reported ^{31}P 2D-chemical exchange studies involving solids measured spin diffusion in single crystals (Connor et al., 1985) or in powders motionally narrowed by MAS (Clayden, 1986). Thus, membrane systems, in which the lipid molecules undergo a complex set of motions over a wide range of frequencies, represent a potentially fruitful area of study for 2D techniques.

In the present study, we demonstrate the utility of ^{31}P 2D-exchange NMR in studying slow motions in phospholipid model systems, and highlight the potential usefulness of the technique for the study of intact biological membranes. The longer T_1 of phosphorus extends the time scales which can be probed relative to those available through deuteron NMR, and the high natural abundance and sensitivity remove the necessity for isotopic enrichment. By way of illustration, we demonstrate orientational exchange via lateral diffusion over the curved surfaces of liquid crystalline (L_α) and gel state ($L_{\beta'}$) phospholipid bilayers, and characterize the time scales and correlation times of this process, thereby allowing estimates of the lateral-diffusion coefficients D_t to be obtained. We then demonstrate orientational exchange in the $P_{\beta'}$ phase of DPPC[1] multilamellar dispersions and oriented samples, and discuss the results in terms of surface rippling and defect diffusion. Finally, preliminary results on two biological systems, the pig erythrocyte ghost and pig spinal cord, representing the membranes of isolated cells and intact tissue, respectively, are presented. Possible applications of the technique are discussed.

MATERIALS AND METHODS

L-α-Dimyristoyl phosphatidylcholine (DMPC) and L-α-dipalmitoyl phosphatidylcholine (DPPC) were obtained from Avanti Polar Lipids, Inc., Birmingham, AL. 1-Palmitoyl-2-oleoyl phosphatidylethanolamine was obtained from Sigma Chemical Co., St. Louis, MO.

Multilamellar dispersions were prepared for NMR by hydrating the lipid with a threefold excess of deionized distilled water, and cyclically heating above the gel to liquid-crystalline phase transition temperature with vortex mixing and freeze-thawing to homogeneity (typically five cycles). Oriented samples were prepared essentially as described by Jarrell et al. (1987) using method B and 10–30 mg of phospholipid.

^{31}P NMR spectra were acquired at 121.5 MHz on a MSL-300 spectrometer (Bruker Instruments, Inc., Billerica MA). One-dimensional spectra were recorded using a Hahn echo pulse sequence (Rance and Byrd, 1983) with WALTZ decoupling (gated on during acquisition). The ^{31}P $\pi/2$ pulse length was 4.0 μs (10-mm solenoid coil), the pulse spacing was 60 μs, and the recycle time was 5.0 s. Two-dimensional spectra were recorded using the basic NOESY pulse sequence with TPPI (used on Bruker spectrometers) to give quadrature detection in both dimensions (Bodenhausen et al., 1984):

$$[(\text{preparation}) - 90° - t_1(\text{evolution}) - 90° - t_{mix} - 90° - t_2(\text{detection}) - (\text{delay})].$$

WALTZ ^1H-decoupling was gated on during the evolution and detection periods. The preparation time was either 32 or 48 s, t_{mix} varied from 100 μs to 200 ms, and the recycle delay was either 2 or 3 s. The data sets were 256 points in the F_2 dimension, and 64 points zerofilled to 256 points in the F_1 dimension. Between 64 and 256 transients were recorded for each serial file in a given 2D experiment. For most experiments, the sweep width in both dimensions was 50 kHz. Other parameters were as described for the 1D experiments.

Spectral simulations

In multilamellar phospholipid systems exhibiting axially symmetric motion, the ^{31}P NMR resonance frequency relative to the isotropic chemical shift is given by (Seelig, 1978)

$$\nu(\beta) = (2/3)\Delta\sigma[(3\cos^2\beta - 1)/2], \quad (1)$$

where $\Delta\sigma$ is the residual chemical shielding anisotropy and β is the angle between the bilayer normal and the magnetic field direction. In the case of lateral diffusion in liposomal systems, lipid molecules move between domains with an associated change in the local bilayer normal orientation relative to the magnetic field direction from β_1 to β_2. As a result of this reorientational process there is an associated change in the ^{31}P resonance frequency. If the timescale of the exchange process is sufficiently slow ($\ll [\nu(\beta_1) - \nu(\beta_2)]^{-1}$), 2D-exchange NMR spectroscopy can provide a means of probing such processes. In a 2D-exchange NMR experiment (given by the pulse sequence $90° - t_1 - 90° - t_{mix} - 90° - \text{detect}[t_2]$) the 2D-absorption mode spectrum is related to the joint probability density $S(\nu_1, \nu_2; t_{mix})$ of having frequency ν_1 during the evolution period t_1 and the frequency ν_2 during the detection period t_2 (Wefing and Spiess, 1988; Wefing et al., 1988). By varying the exchange time, t_{mix}, the timescale and mechanism of molecular reorientation (in this case lateral diffusion) may be quantitated. The two-dimensional time domain signal is given by (Wefing et al., 1988)

$$S_p(t_1, t_2; t_{mix}) = \int d\beta_1 \int d\beta_2 p[\nu(\beta_1)t_1] \times [\exp(-i\nu(\beta_2)t_2)] \times \Gamma(\beta_1, \beta_2; t_{mix}), \quad (2)$$

where the integration is performed between 0 and $\pi/2$ radians, and p is cos and sin representing the two data sets required for the absorption mode spectrum obtained after Fourier transformation according to the scheme of States and co-workers (States et al., 1982). For lateral diffusion (represented as isotropic rotational diffusion with a correlation time $\tau_d = 1/6D_t$, where D_t is the diffusion constant) the joint probability $\Gamma(\beta_1, \beta_2; t_{mix})$ was calculated as outlined by Spiess and co-workers (Wefing et al., 1988) using a discrete approximation of the continuous distribution of angles β and using standard computer library routines. ^{31}P 2D-exchange spectra (both the cosine and sine data sets) were calculated according to Eq. 2 using 3° steps in the β_1 and β_2 angular ranges (this corresponds to a 60 × 60 matrix for Γ). Spectra were simulated as a function of the ratio t_{mix}/τ_e (where τ_e is the effective correlation time defined below; see Eq. 4) with 64 values of t_1, and 256 values of t_2 such that the sweep width in both dimensions was 50 kHz. An angular dependent transverse relaxation rate of the form

$$1/T_2(\beta) = A + B|3\cos^2\beta - 1|/2 \quad (3)$$

[1]*Abbreviations used in this paper:* DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; FRAP, fluorescence recovery after photobleaching; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine.

(leading to a corresponding angular dependent Lorentzian linewidth in the frequency spectra) was used in each of the t_1 and t_2 domains to better approximate the experimental linewidth behavior (Seelig, 1978; Larsen et al., 1987). Spectra were zero filled before Fourier transformation to give a 256 × 256 2D spectrum. All spectral simulations were performed on a Sun4-260 computer and processed using NMR2 software (New Methods Research Inc., Syracuse, NY).

RESULTS

Lateral diffusion in liquid-crystalline multilamellar dispersions and oriented samples

The 2D exchange experiment, first described by Jeener et al. (1979), measures chemical (hereafter referred to as orientational) exchange or spin diffusion in a given mixing period. The pulse sequences used to record two-dimensional spin-1/2 exchange spectra of solids are the same or analagous to those employed in high-resolution 2D experiments in liquids (Hagemeyer et al., 1989). In the present case, we have used the basic NOESY pulse sequence with WALTZ decoupling. The first 90° pulse creates transverse magnetization which is frequency labeled during the evolution period. The second 90° pulse creates z magnetization; exchange processes occur during this mixing period t_{mix}. The third 90° pulse again creates transverse magnetization, in which the exchanged components are now precessing at their new frequencies. The experiment is repeated by incrementing the evolution time, and a double Fourier transform is performed. Components which did not undergo orientational exchange appear along the diagonal, which in this case is the ^{31}P powder pattern lineshape. The presence of off-diagonal intensity indicates exchange between the orientations represented by the connected frequencies. The diffusion of phospholipid molecules over the curved surfaces of liposomes is an ideal example of orientational exchange with which to demonstrate the usefulness of the 2D method. The ^{31}P powder pattern results from the angular anisotropy of the chemical shielding interaction and is broadened by ^{31}P-^{1}H dipolar interactions; each frequency of the spectrum corresponds to a different angle β between the bilayer normal and the magnetic field. Thus, when a molecule moves from one local bilayer normal having orientation β_1, to another having orientation β_1, there is a change in the associated frequency. 'Holeburning' experiments (using the DANTE pulse sequence) have demonstrated that lateral diffusion can transfer magnetization from one frequency to another in the millisecond time scale (short with respect to the ^{31}P T_1) (Larsen et al., 1987; Milburn and Jeffrey, 1987). This exchange between frequencies is readily visualized in a single 2D experiment. Fig. 1 shows a series of 2D experiments on DMPC at 30°C. The stacked plots in the left column are the experimental spectra, and the plots in the right column are simulations. The corresponding contour plots are shown in Fig. 2. The top left spectrum (Figs. 1 and 2) was obtained using t_{mix} = 1 ms; in this short time period little change in β occurs, and all the intensity is located on the diagonal. As t_{mix} is increased to 10 ms (*center left*), significant off-diagonal intensity is observed, and it is readily seen that the frequency associated with β = 90° is connected with frequencies near the center of the powder pattern and approaching β = 0° (indicating orientational exchange between β = 90 and 0°). As the mixing time is further increased to 100 ms, the off-diagonal intensity increases (*bottom left*). There is little

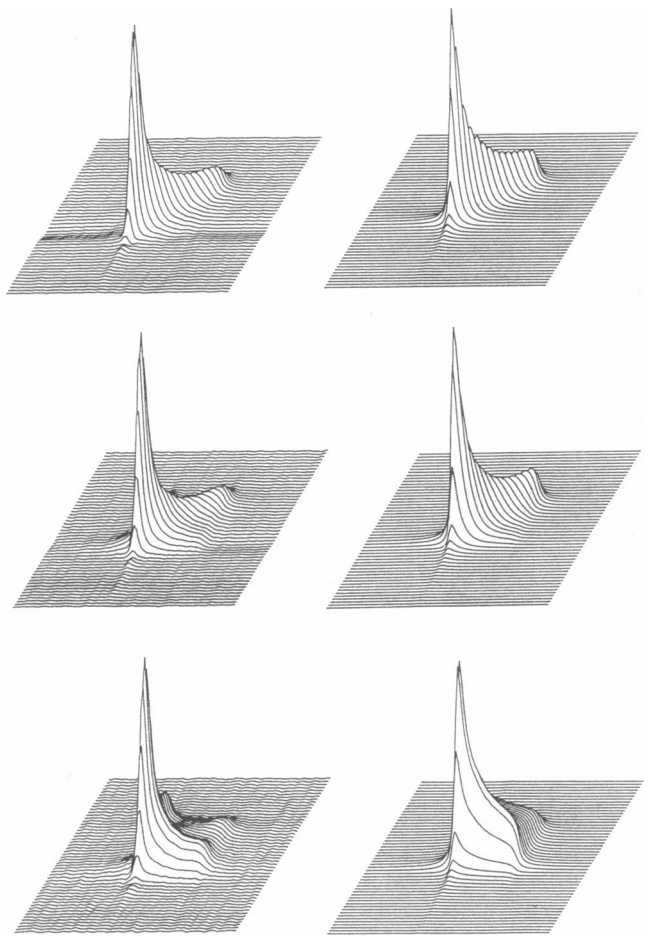

FIGURE 1 2D-exchange spectra of DMPC aqueous multilamellar dispersions at 30°C (*left column*) for t_{mix} = 1 (*top*), 10 (*center*), and 100 ms (*bottom*). The simulations (*right column*) were performed as described in the text using t_{mix}/τ_d = 0.02 (*top*) and 3 (*bottom*). The spectrum in the center is 20% t_{mix}/τ_d = 1.5 and 80% t_{mix}/τ_d = 0.1. The plot width is ±10 kHz in both dimensions.

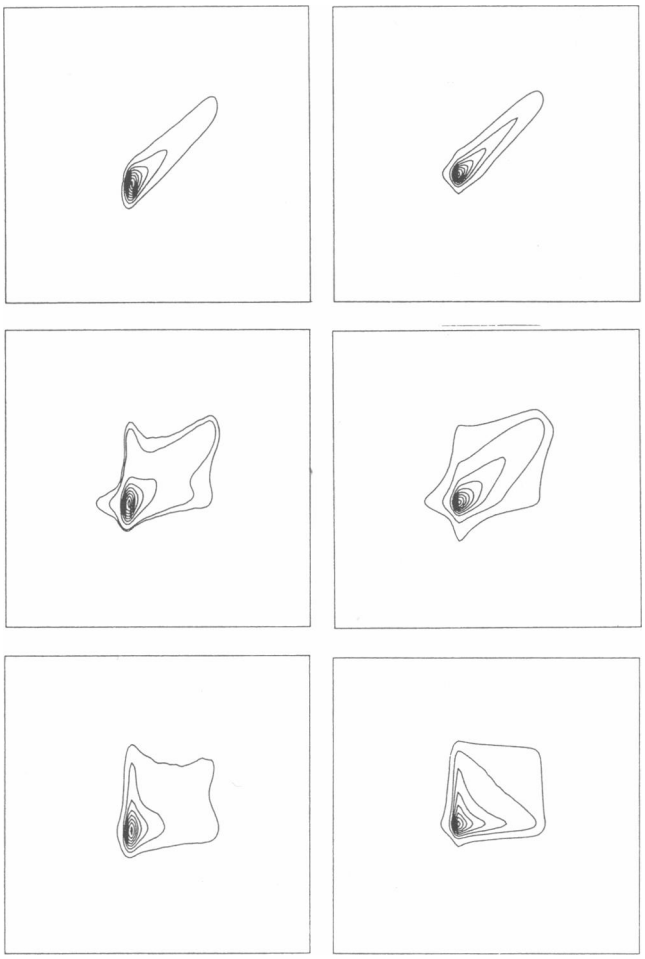

FIGURE 2 Corresponding contour plots of the spectra in Fig. 1 (DMPC at 30°C). The plot width is ±10 kHz in both dimensions.

change between t_{mix} = 100 and 200 ms (not shown), other than a slight increase in the off-diagonal intensity, indicating that the system is fully exchanged in a period of ~100 ms.

The simulations in Figs. 1 and 2 were performed as described in Materials and Methods, using $\Delta\sigma$ = 46 ppm and values of A and B (Eq. 3) of 50 and 200 Hz, respectively (Larsen et al., 1987). During processing, a line broadening of 300 Hz followed by apodization with a $\sin^2(\theta)$ function (where $\theta = (i - 1)\pi/(SIZE - 1)$, where *SIZE* is the number of data points in the current dimension and i = 1, 2, 3, . . . *SIZE*) (used in the processing of the experimental spectra) was applied in both time domains before Fourier transformation. A series of simulated spectra were calculated for t_{mix}/τ_e values ranging from 0–50, which were then matched with experimental spectra. It is clear that the simulated and experimental lineshapes are not identical, which may indicate that exchange processes are occurring during the evolution and detection periods; the simulation program does not account for such possible effects. Nevertheless, the agreement between prediction and experiment is very good, and all the essential features of the experimental spectra are reproduced by the simulations. This allows us to obtain estimates of the effective correlation time for orientational exchange, τ_e, for a given phospholipid at a given temperature. The effective reorientational rate $1/\tau_e$ for a phospholipid in a liposome is related to the rates of liposome tumbling $1/\tau_t$ and phospholipid lateral diffusion $1/\tau_d$ by

$$1/\tau_e = 1/\tau_t + 1/\tau_d, \quad (4)$$

where

$$\tau_t = 4\pi\eta R^3/3kT \quad (5)$$

$$\tau_d = R^2/6D_t, \quad (6)$$

where η is the solvent viscosity, k is the Boltzmann constant, T is the absolute temperature, and R is the radius of the liposome. Based on estimates of the effective or mean radius of liposome preparations, which range from 1 μm (Larsen et al., 1987) to 1.2 μm (Sternin, 1988), we can neglect the overall tumbling of the liposome as a source of the observed exchange in the liquid-crystalline phase; for R = 1–1.2 μm, τ_t = 0.8–1.4 s. As seen below, the correlation times for exchange for fluid DMPC and DPPC are <50 ms, thus, $1/\tau_d \gg 1/\tau_t$, and $\tau_e = \tau_d$. To obtain quantitative measurements of D_t, one would have to know the liposomal size distributions, which would allow spectra to be simulated for a distribution of correlation times corresponding to the distribution of liposome radii. This is beyond the scope of the present paper.

For the spectra in Figs. 1 and 2 with t_{mix} = 1 and 100 ms, reasonable fits were obtained using t_{mix}/τ_d = 0.02 and 3, giving τ_d = 50 and 33 ms, respectively. For t_{mix} = 200 ms, the best fit was obtained for t_{mix}/τ_d = 4, giving τ_d = 50 ms. However, the spectrum in Figs. 1 and 2 with t_{mix} = 10 ms was not adequately reproduced by any of the simulations. The majority of the intensity is located along the diagonal, but a small yet significant proportion of off-diagonal intensity connects the 90 and 0° orientations. The spectrum could only be simulated by varying the proportions of two simulations for significantly different values of t_{mix}/τ_d. One such solution is shown in Figs. 1 *b* and 2 *b*, which represent 20% of t_{mix}/τ_d = 1.5, and 80% of t_{mix}/τ_d = 0.1, for which τ_d = 7 and 100 ms, respectively. This suggests that some heterogeneity is present in the multilamellar dispersions. This may indicate that there are different populations of diffusing lipid, or, more likely, the phospholipid with the shorter τ_d may originate

from smaller structures, where reorientational effects would be observed over a shorter timescale. It should be stressed that the simulation in Figs. 1 and 2 (for t_{mix} = 10 ms) does not represent a unique solution, and for this reason we have not included it in the estimation of τ_d. A similar treatment of the other spectra has not been attempted for the same reason. Furthermore, the faster exchanging component represents a small proportion of the total phospholipid, which would be difficult to detect at longer mixing times. The simulations indicate that the greatest changes are observed at smaller ratios of t_{mix}/τ_d, in the range of 0–1. By averaging the τ_d values obtained from the best fit simulations for t_{mix} = 1, 100, and 200 ms, we obtain τ_d = 44 ms for DMPC.

Similar experiments were performed on DPPC at 45°C; the contour plots of experimental spectra and simulations are shown in Fig. 3. It is apparent from the spectra that the rate of orientational exchange is greater in DPPC, as significant exchange is observed at t_{mix} = 1 ms, for which little or none was observed for DMPC. As for DMPC with t_{mix} = 10 ms, this spectrum was best simulated with a sum of t_{mix}/τ_d ratios; the simulation shown corresponds to 50% t_{mix}/τ_d = 0.125 and 50% t_{mix}/τ_d = 1.5, giving τ_d = 8 and 0.7 ms, respectively. Again, this cannot be considered to be a unique solution, because the latter result suggests, as was the case with DMPC, that there is a distribution of liposome sizes and that the spectra should be represented by a corresponding distribution of τ_d values. The best single ratio fit, which was clearly inferior, was obtained for t_{mix}/τ_d = 0.4. Very good simulations of the other spectra were obtained using a single ratio of t_{mix}/τ_d (0.05, 1.0, 10.0, and 20.0 for t_{mix} = 0.1, 10, 100, and 200 ms, respectively), giving a mean τ_d of 8 ms. Thus, the lateral diffusion rate of DPPC is greater than for DMPC at comparable reduced temperatures.

The presence of orientational exchange via lateral diffusion in the powder samples is further verified by experiments performed on oriented samples. POPE and DPPC were macroscopically oriented between glass plates, and spectra were acquired at an angle of 55° between the normal to the bilayer surface and the external magnetic field (the "magic angle"). This bilayer orientation has the property that the resonance frequency is the most sensitive to changes in β (see Eq. 1). Thus, even diffusion over bilayers of low curvature should be detectable. Fig. 4, *top* and *bottom*, show spectra of POPE at 30°C for t_{mix} values of 1 and 100 ms, respectively; the two spectra are essentially identical. Because the oriented bilayers are planar, lateral diffusion does not change the angle between the magnetic field direction and the bilayer normal, and thus there is no slow orientational exchange in these systems. The same was observed for oriented bilayers of DPPC at 45°C for t_{mix} = 50 ms (not shown).

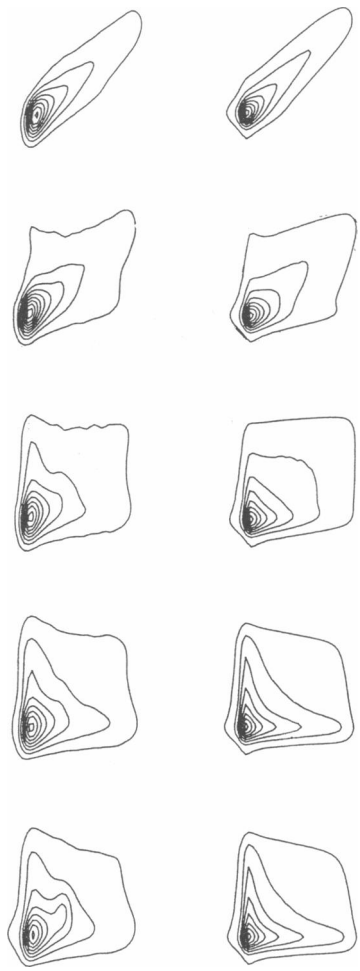

FIGURE 3 2D-exchange spectra of DPPC aqueous multilamellar dispersions at 45°C (*left column*) for t_{mix} = 0.1, 1, 10, 100, and 200 ms (*top to bottom*). The simulations (*right column*) were performed as described in the text using t_{mix}/τ_d = 0.05, 0.5(1.5) + 0.5(0.125), 1, 10, and 20 (*top to bottom*). The scale is approximately the same as in Fig. 2.

Investigation of POPE multilamellar dispersions at 30°C (spectra not shown) revealed little exchange for t_{mix} = 1 ms, and essentially complete exchange for $t_{mix} \geq$ 20 ms.

Detection of orientational exchange in the $L_{\beta'}$ and $P_{\beta'}$ phases of DPPC

At 30°C, DPPC multilamellar dispersions exist in the $L_{\beta'}$ phase, where the rate of lateral diffusion is reduced, relative to the L_α phase, by at least two orders of magnitude (Wu et al., 1977; Vaz et al., 1982; Schneider et al., 1983; Kapitza et al., 1984). Thus, the time scale for

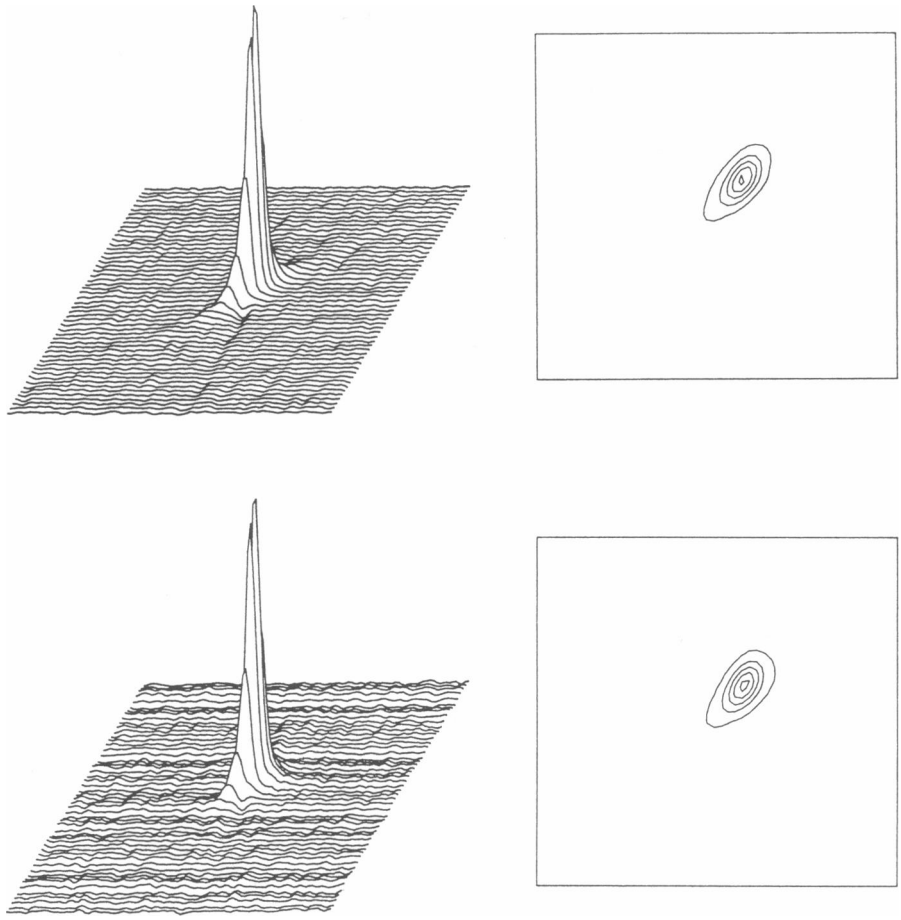

FIGURE 4 2D-exchange spectra of POPE oriented multibilayers at 30°C for t_{mix} = 1 (*top*) and 100 ms (*bottom*). The angle between the bilayer normal and the magnetic field was 55°. The plot widths of the stacked and contour plots are ±2 and ±1 kHz in both dimensions, respectively.

exchange should be significantly longer than for liquid-crystalline bilayers. Fig. 5 shows experimental (*left column*) and simulated (*right column*) spectra for DPPC bilayers at 30°C, for various values of t_{mix}. The stacked plot at the top of the figure was obtained with t_{mix} = 1 ms, and the contour plots at the bottom were obtained with t_{mix} = 1, 50, and 200 ms (*top to bottom*). The simulations were performed using an axially symmetric lineshape with $\Delta\sigma$ = 69 ppm, and an orientation-dependent linewidth with A = 500 Hz and B = 500 Hz (Eq. 3). During processing, a Gaussian multiplication with 1 kHz linewidth followed by apodization with a $\sin^2(\theta)$ function (as described earlier for the liquid-crystalline simulations) were applied in both dimensions before Fourier transformation. Whereas the simulations do not precisely reproduce the gel-state lineshape, they do allow the effects of slow lateral diffusion to be probed. For t_{mix} = 1 ms, all of the spectral intensity is located on the diagonal. As t_{mix} is increased to 50 and 200 ms, the width of the powder pattern is increased, and the increase in width is fairly uniform throughout the powder pattern. This increase in width is also observed in the simulations, which correspond to t_{mix}/τ_e ratios of 0.01, 0.075, and 0.175, going from top to bottom. This corresponds to a τ_e of ~900 ms. In this case the correlation time obtained from the simulations is of the same magnitude as τ_t, the correlation time for liposomal tumbling, for particles with radii in the 1 μm range. Thus, the observed exchange likely results from a combination of liposomal tumbling and lateral diffusion, which makes accurate knowledge of the size distribution a necessity if an estimate of D_t is desired. It is clear, however, that orientational exchange in the gel state is at least two orders of magnitude slower than in the liquid-crystalline phase.

DPPC exists in the $P_{\beta'}$ phase between 34 and 42°C. Several 2D spectra were recorded in this temperature interval for t_{mix} values of 1 and 10 ms (not shown). The

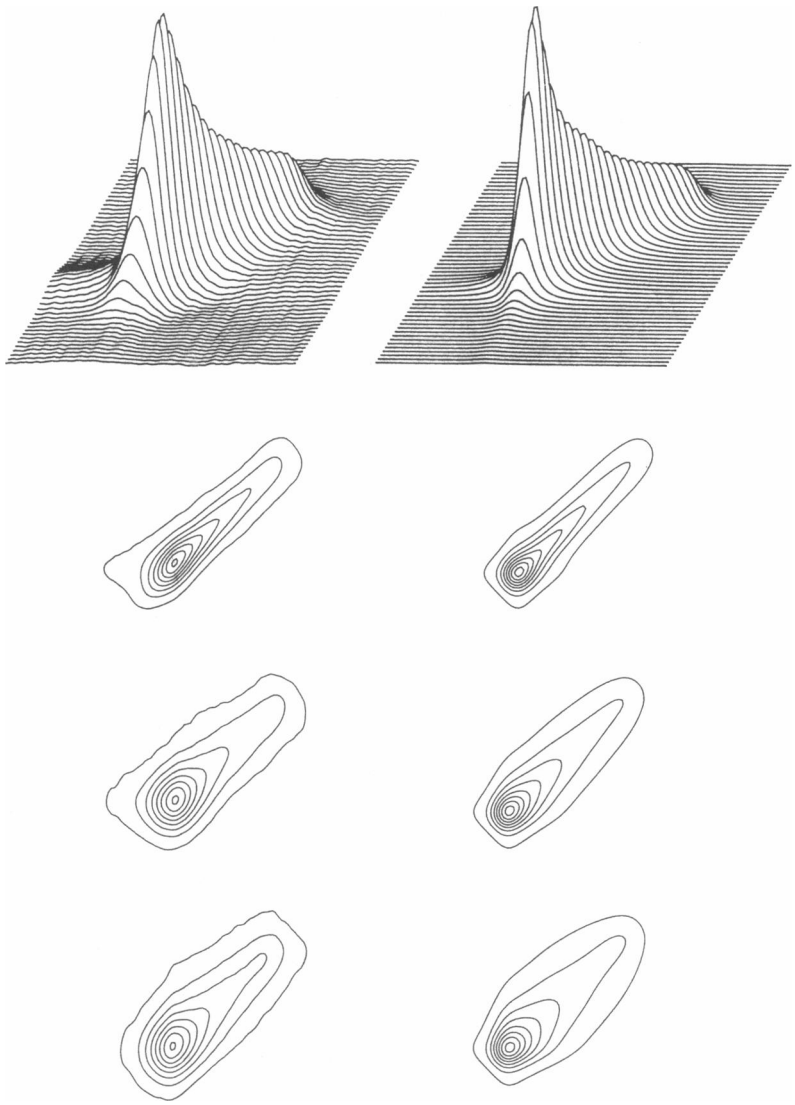

FIGURE 5 2D-exchange spectra (*left column*) of DPPC aqueous multilamellar dispersions at 30°C ($L_{\beta'}$ phase) for t_{mix} = 1 ms (*stacked plot*). The contour plots correspond to t_{mix} = 1 (*top*), 50 (*center*), and 200 ms (*bottom*). The simulations (*right column*) were performed as described in the text and correspond to t_{mix}/τ_e values of 0.01 (*stacked plot and top contour plot*), 0.075 (*center contour*), and 0.175 (*bottom contour*). The plot width of the stacked plot is ±10 kHz in both dimensions and the scale of the contour plots is approximately the same.

contour plots obtained between 34 and 39°C for t_{mix} = 1 ms are similar to that obtained at 30°C for the same mixing time in that no off-diagonal intensity is observed. At 39°C for t_{mix} = 10 ms, there is an increase in the width of the powder pattern and a small amount of off-diagonal intensity.

The amount of off-diagonal intensity at 39°C is greatly increased if t_{mix} is increased to 50 ms. This is shown in Fig. 6, where several spectra were obtained as the temperature was decreased from 39 to 30°C. The spectra show little change between 39 and 35°C, but there is a slight decrease in off-diagonal intensity as the temperature is cooled to 33°C. The off-diagonal intensity disappears as the temperature is further cooled to 32°C, where the 2D spectrum is essentially identical to the 30°C spectrum. This is observed 2°C lower than the $P_{\beta'}$ to $L_{\beta'}$ phase-transition temperature of 34°C, and may result from hysteresis in the phase transition, even though the samples were equilibrated for 1–1.5 h between runs. Davis (1979) observed a 1°C hystersis in the main gel to liquid-crystalline phase transition of DPPC. Thus, the observed orientational exchange appears to be associated exclusively with the $P_{\beta'}$ phase. This was verified by increasing the temperature from 32 to 38°C after an

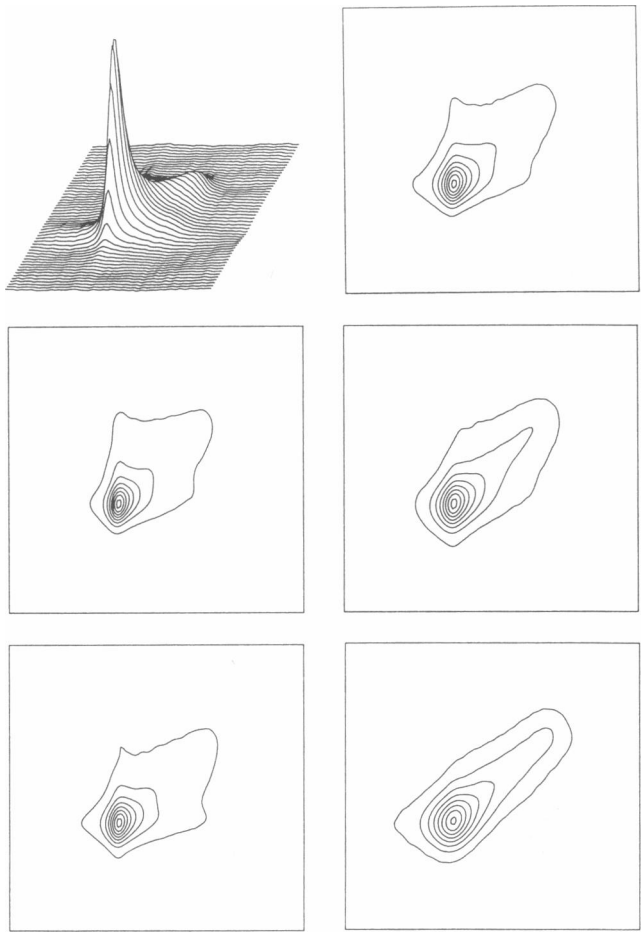

FIGURE 6 Stacked plot of 2D-exchange spectrum of DPPC multilamellar dispersion obtained at 39°C with t_{mix} = 50 ms (*top left*). Contour plots of 2D-exchange spectra of DPPC multilamellar dispersions obtained between 39°C ($P_{\beta'}$ phase) and 30°C ($L_{\beta'}$ phase) for t_{mix} = 50 ms (cooling run). Temperatures = 39°C (*left center*), 37°C (*left bottom*), 35°C (*right top*), 33°C (*right center*), and 32°C (*right bottom*). The plot width is ±10 kHz in both dimensions.

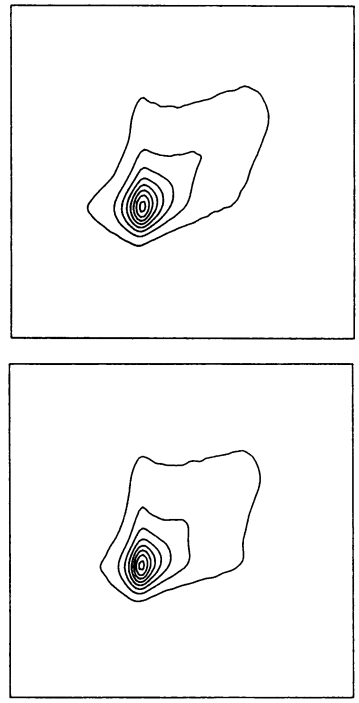

FIGURE 7 Contour plots of 2D-exchange spectra of DPPC multilamellar dispersions obtained at 34°C (*top*) and 36°C (*bottom*) ($P_{\beta'}$ phase) for t_{mix} = 200 ms (heating run). The plot width is ±10 kHz in both dimensions.

extended equilibration at 30°C. For t_{mix} = 50 ms, no orientational exchange was observed at 30 or 32°C. At 34°C, the $L_{\beta'}$ to $P_{\beta'}$ phase-transition temperature, the exchange became noticeable, and increased slightly as the temperature was raised to 36°C and then to 38°C (not shown). Significant differences are also observed between the $L_{\beta'}$ and $P_{\beta'}$ spectra for t_{mix} = 200 ms. Fig. 7 shows spectra obtained at 34 and 36°C, which should be compared with the corresponding spectrum for 30°C in Fig. 5 (*bottom left*). At comparable temperatures, the exchange is somewhat greater for t_{mix} = 200 ms than t_{mix} = 50 ms.

Another difference between the $L_{\beta'}$ and $P_{\beta'}$ phases is the lineshape of the ^{31}P powder spectrum, which can be seen clearly in the stacked plots in Fig. 5 and in Fig. 6. The intensity of the 0° shoulder relative to the 90° shoulder is greatly reduced in the ripple phase.

Two-dimensional spectra of DPPC in the $L_{\beta'}$ and $P_{\beta'}$ phases were also obtained using oriented samples. One-dimensional spectra of the oriented samples were initially acquired at 45°C (liquid-crystalline phase), at the 0° orientation, to estimate the presence, if any, of unoriented phospholipid. A small amount could be observed at the 0° orientation, but not at the 55° orientation. A stacked plot of DPPC at 30°C (t_{mix} = 50 ms), oriented at an angle of 55° between the normal to the bilayer and the magnetic field, is shown in Fig. 8. The gel phase oriented spectrum is very broad compared with that observed in the L_{α} phase. Several contour plots for oriented spectra in the temperature range of 30 to 39°C are shown in Fig. 9. There is a definite change in the shape of the countour plots in the $P_{\beta'}$ phase. At 30°C, the contour plot is an oval shape, whereas at 37 and 39°C, an increase in off-diagonal intensity is observed near the center of the spectrum. At 39°C, for t_{mix} = 1 ms, this off-diagonal intensity is completely removed (Fig. 9, *bottom right*). Thus, it is clear that some orientational exchange is being detected in the $P_{\beta'}$ phase of the oriented sample.

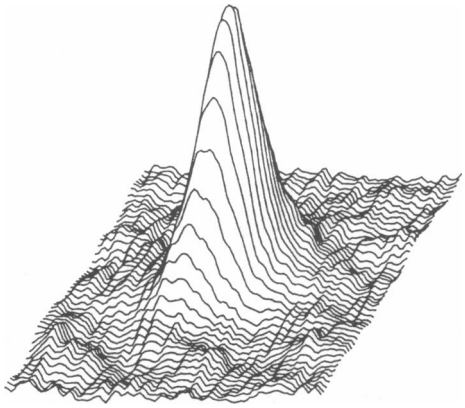

FIGURE 8 Stacked plot of 2D exchange spectrum of DPPC oriented multibilayers obtained at 30°C ($L_{\beta'}$ phase) for t_{mix} = 50 ms. The angle between the bilayer normal and the magnetic field was 55°. The plot width is ±10 kHz in both dimensions.

Detection of orientational exchange in biological membranes

Two biological systems were chosen for comparison with the model systems, these being pig erythrocyte ghost membranes, as an example of isolated cells, and pig spinal cord, an intact tissue. Stacked and contour plots of the pig erythrocyte ghosts are shown in Fig. 10. At t_{mix} = 100 ms, there is a hint of off-diagonal intensity, which is significant by t_{mix} = 200 ms. Unfortunately, the signal-to-noise ratio was very poor in these preparations for the longer mixing times. Nevertheless, it is clear that significant exchange can be observed in 200 ms. The spectra obtained for t_{mix} = 100 and 200 ms are reasonably simulated using t_{mix}/τ_d = 0.2 and 0.6, respectively (not shown). This gives an average τ_d of ~400 ms. Different behavior was observed with the pig spinal cord. Stacked and contour plots of spectra obtained at 30°C are shown in Fig. 11. For t_{mix} = 1 ms, all the intensity is located on the diagonal, as observed in the model system. Similar results were obtained for mixing times of 5 and 20 ms (not shown). Even at mixing times of 100 and 200 ms, only a slight broadening and a small amount of off-diagonal intensity is detected. These spectra were best

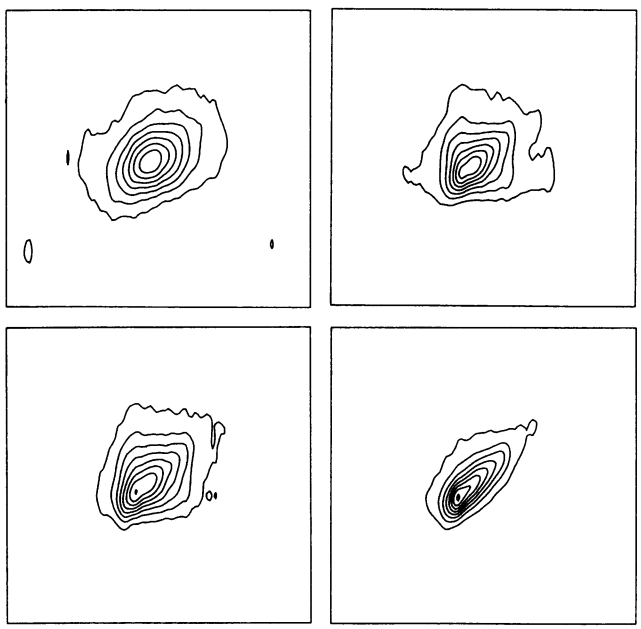

FIGURE 9 Contour plots of 2D-exchange spectra of DPPC oriented multibilayers obtained at 30°C ($L_{\beta'}$ phase) (*top left*), 37° ($P_{\beta'}$ phase) (*bottom left*), and 39°C ($P_{\beta'}$ phase) (*top right*) for t_{mix} = 50 ms, and at 39°C for t_{mix} = 1 ms (*bottom right*). The angle between the bilayer normal and the magnetic field was 55°. The plot width is ±10 kHz in both dimensions.

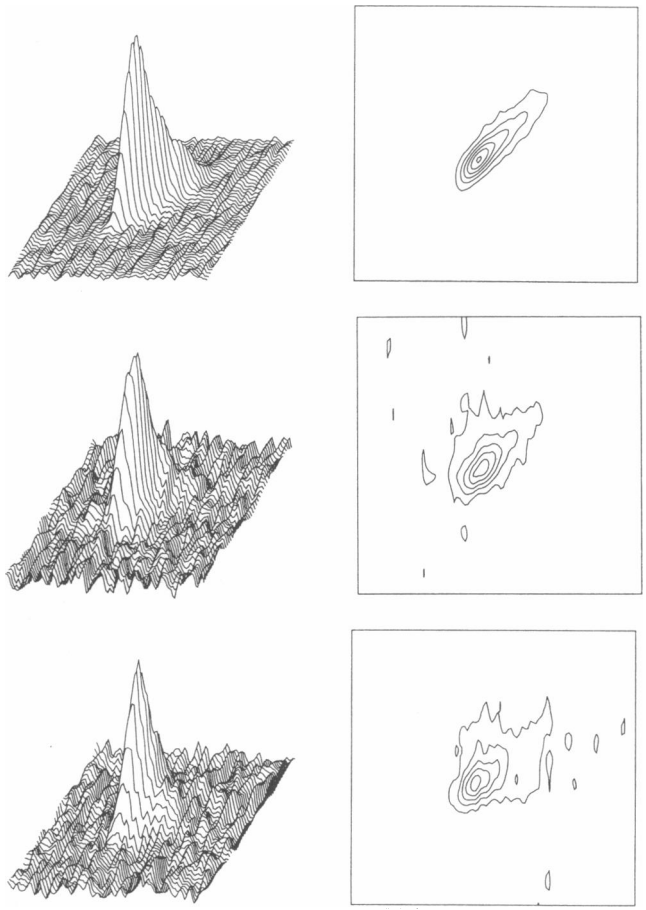

FIGURE 10 Stacked (*left column*) and contour (*right column*) plots of 2D exchange spectra of pig erythrocyte ghosts obtained at 30°C for t_{mix} = 1 (*top*), 100 (*center*), and 200 ms (*bottom*). The plot width is ±10 kHz in both dimensions.

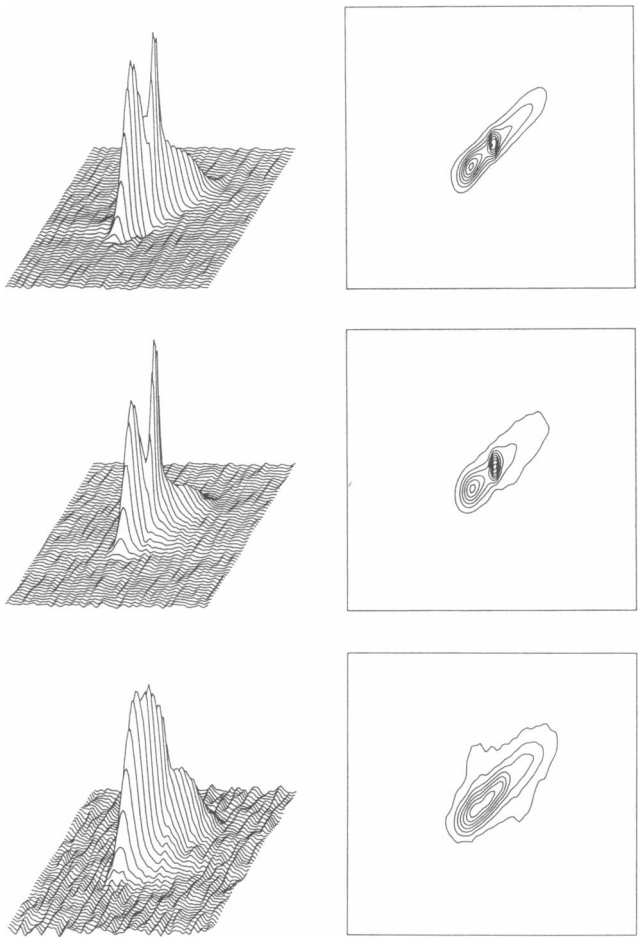

FIGURE 11 Stacked (*left column*) and contour (*right column*) plots of 2D-exchange spectra of pig spinal cord obtained at 30°C for t_{mix} = 1 (*top*), 100 (*center*), and 200 ms (*bottom*). The plot width is ±10 kHz in both dimensions.

simulated using t_{mix}/τ_d = 0.1 and 0.125, respectively, giving an average τ_d of ~1.3 s; thus, exchange in the spinal cord appears to occur over a much longer time period than in the erythrocytes. Furthermore, the time scale of exchange appears to be significantly longer in these biological membranes than in model systems.

DISCUSSION

The present paper establishes the application of solid-state 2D-orientational exchange ^{31}P NMR to problems of membrane structure. The technique allows the approximate time scales of slow diffusional processes to be easily and quickly determined, and may provide a method for detecting membrane surface distortions. Furthermore, if the mean size or size distribution of the particles under investigation is known, estimates of the lateral-diffusion coefficients of phospholipids in both liquid-crystalline and gel-state membranes should be obtainable.

Orientational exchange in liquid-crystalline bilayers

The results obtained with multilamellar dispersions of DPPC, DMPC, and POPE in the L_α phase demonstrate orientational exchange via lateral diffusion over the curved surfaces of liposomes. The observed off-diagonal intensity correlates different frequencies which represent phospholipid molecules oriented at different angles β_i with respect to the external magnetic field. Our results demonstrate that significant exchange occurs on the millisecond timescale. In the case of DPPC, lateral diffusion can be detected in the range of 1–10 ms, whereas for DMPC, similar exchange is detected between 10 and 100 ms. Spectral simulations allow us to estimate the correlation time τ_d for diffusion to be 44 ms for DMPC at 30°C and 8 ms for DPPC at 45°C. These values are in excellent ageement with those obtained using ^{31}P NMR by Larsen et al. (1987) (33 ms) and Milburn and Jeffrey (1987) (10 ms) for egg PC using the DANTE pulse sequence, but are somewhat shorter than obtained by Sternin (1988) (62 ms) using a form of the Carr-Purcell-Meiboom-Gill pulse sequence modified for ^2H NMR (Bloom and Sternin, 1987). Recently, the orientational exchange observed for the terminal methyl groups of perdeuteriated DPPC was measured by 2D solid-state deuteron NMR, and a τ_d value of 10 ms was obtained (Auger, M., I. C. P. Smith, and H. C. Jarrell, unpublished results), essentially identical to the value obtained here. For liposomes of similar size and shape, our results indicate faster lateral diffusion of DPPC at 45°C than of DMPC at 30°C.

Although quantitative determination of lateral-diffusion coefficients D_t requires independent knowledge of the size distribution of our liposome preparations, it would be instructive to have a rough estimate of the diffusion rates obtained by the 2D method for comparison with other techniques. Other workers have assumed (Larsen et al., 1987) or estimated via ^2H NMR and light scattering (Sternin, 1988) an effective liposome radius in the range of 1–1.2 μm. We have no reason to believe that the mean sizes of our particles would be significantly different. Thus, assuming R = 1.2 μm (Sternin, 1988), estimates of the lateral-diffusion coefficients D_t were obtained for DMPC (4×10^{-8} cm^2/s) and DPPC (3×10^{-7} cm^2/s) using Eq. 6. The lateral-diffusion coefficients of phospholipids in both model and biological membranes have been measured by a variety of techniques; the most widely used is fluorescence recovery after photobleach-

ing (FRAP) (Vaz et al., 1982). In the liquid-crystalline phase, D_t values on the order of 10^{-8} to 10^{-7} cm^2/s have routinely been measured, in agreement with values obtained by other methods such as NMR (Mackay et al., 1978 and references therein). Vaz et al. (1985) used FRAP to study the diffusion of NBD-PE in a variety of liquid-crystalline phosphatidylcholine bilayers, and found that the diffusion of DPPC is faster than diffusion of DMPC at comparable reduced temperatures. At 30°C, D_t for DMPC was $\cong 6 \times 10^{-8}$ cm^2/s, and at 45°C, D_t for DPPC was $\cong 1.3 \times 10^{-7}$ cm^2/s. Considering the approximate nature of the D_t values calculated above, the agreement between 2D NMR and FRAP is very good. Thus, the correlation times for lateral diffusion obtained in the present study are consistent with measured diffusion rates, assuming liposome radii in the 1 μm range. Further refinement of the technique, either by simulations which incorporate particle size distributions, or by the use of lipid preparations with a narrow and well-defined size distribution, should allow the quantitative measurement of D_t values. An advantage of the 2D NMR technique is that it is possible to study unmodified lipids; one does not have to be concerned about the effects of bulky fluorescent reporter groups. This may be of particular importance in the gel state or in more complicated membranes where phase separation of some components is a possibility.

That the exchange observed in the multilamellar dispersions is due to lateral diffusion alone is clearly demonstrated by the use of macroscopically oriented samples. In this case, the angle β between the membrane surface normal and the magnetic field is unchanged by lateral diffusion if the surface is not distorted over long distances, and no exchange is observed at mixing times as long as 100 ms (Fig. 4). That the effects of diffusion can be thus removed is an important point, for any orientational exchange observed in an oriented sample must then originate from modulations of the angle between the membrane normal and magnetic field caused by local perturbations to the membrane surface. In principle, 2D orientational exchange NMR could be used to provide information on any process which distorts the topography of membranes or restricts the lateral mobility of lipids. One possibility which we are currently investigating is the lamellar to hexagonal phase transition observed in many PEs. Another system which may prove quite interesting is the intermediate "rippled" phase of DOPC which occurs at reduced hydration between the L_α and H_{II} phases (Bradshaw et al., 1989). The ripples are sinusoidal with a wavelength on the order of 6 nm; the derived model predicts angular oscillations over one ripple wavelength of up to 45° with respect to the average membrane normal.

Orientational exchange in gel-state bilayers

In the gel phase of DPPC (30°C), there is little orientational exchange over the time scales investigated in the current paper. As t_{mix} is increased from 1 to 200 ms, the width of the 2D-powder spectrum (i.e., in the direction perpendicular to the diagonal) approximately doubles, but there is no intensity linking the 90 and 0° orientations. By simulating the time evolution of exchange in the gel state, we estimate the correlation time for orientational exchange τ_e to be $\cong$ 900 ms at 30°C. This value is of the same magnitude as τ_t, the correlation time for liposomal tumbling, and thus both lateral diffusion and particle tumbling may contribute to the observed exchange. In this case, an accurate knowledge of the particle size distribution is even more important for determining D_t due to the R^3 term in τ_t (Eq. 5). However, for liposomes with radii on the order of 1.2 μm (Sternin, 1988), $\tau_d \cong 2.7$ s, and $D_t \cong 2 \times 10^{-9}$ cm^2/s. For R values < 1.2 μm, longer τ_d values are obtained, corresponding to slower lateral diffusion. Thus, our results suggest that diffusion in the gel state is $\leq 10^{-9}$ cm^2/s. Previous estimates of D_t in the gel phase obtained by FRAP range between 3×10^{-17} cm^2/s and 10^{-10} cm^2/s (Wu et al., 1977; Fahey and Webb, 1978; Vaz et al., 1982; Schneider et al., 1983; Kapitza et al., 1984). The orientational exchange observed in the gel state should not originate from defect structures, which are detected in the $P_{\beta'}$ phase (see below). <5% of the phospholipid in the gel phase of DMPC ($T < 10$°C) can undergo rapid lateral diffusion as measured by FRAP (Kapitza et al., 1984).

One system where the local packing of the membrane offers the potential for an additional source of orientational exchange is the "rippled" $P_{\beta'}$ phase of gel-state, phospholipids such as DPPC and DMPC. The $P_{\beta'}$ phase occurs between the gel $L_{\beta'}$ and liquid-crystalline L_α phase, and is characterised by the presence of ripples with a wavelength of 14–16 nm (Stamatoff et al., 1982; Alecio et al., 1985; Wack and Webb, 1989). The chains are mostly extended and tilted with respect to the mean membrane normal (Wack and Webb, 1989). Whereas earlier FRAP studies were interpreted in terms of a homogeneous ripple phase characterized by very slow lateral diffusion (Wu et al., 1977; Fahey and Webb, 1978; Vaz et al., 1982), there is now considerable evidence suggesting the existence of a significant population of defect structures, in which are present 10–20% of the membrane lipids (Kapitza et al., 1984). FRAP lateral diffusion measurements of the $P_{\beta'}$ phase of DMPC find two diffusion pathways (Derzko and Jacobson, 1980; Schneider et al., 1983; Kapitza et al., 1984), although there are significant differences in the reported values of D_t for the two populations. Schneider et al. (1983)

reported "slow" diffusion in ordered crystalline domains with $D_s \cong 10^{-16}$–10^{-17} cm²/s, and "fast" diffusion in defect structures with $D_f \cong 4 \times 10^{-11}$ cm²/s. Kapitza et al. (1984) reported that, at low-lipid probe concentrations, ~20% of the probe diffused fast ($D_f = 10^{-8}$–10^{-9} cm²/s), whereas the rest was strongly immobilized ($D_s < 10^{-10}$ cm²/s). The two groups used different fluorescent probes, which may account for the differences. The fast diffusion is thought to occur along linear defects or grain boundaries (Kapitza et al., 1984), the former of which may be associated with the ridges and furrows of the ripples (Schneider et al., 1983). Evidence for the existence of defects in DPPC vesicles comes from the partitioning of amphipaths into the $L_{\beta'}$, $P_{\beta'}$, and L_α phases (Müller et al., 1986). However, it should be noted that a synchrotron x-ray study of the $P_{\beta'}$ phase of lecithin-water systems at less than complete hydration was not consistent with a defect population of >10% (Wack and Webb, 1989).

2D measurements on the $P_{\beta'}$ phase thus may allow detection of orientational exchange from two sources. (*a*) diffusion of phospholipids over the curved ripples, or (*b*) diffusion of lipids within or along disordered defect structures. In principle, both processes may result in changes in the instantaneous orientation of the lipid long axis with respect to the external magnetic field. Nevertheless, it is necessary to note that none of the x-ray diffraction models of the gel ripple phase published to date suggest large angular variations in the mean orientation of phospholipid molecules as they diffuse over the ripples, in contrast to the fluid phase rippling observed in DOPC bilayers of low hydration (Bradshaw et al., 1989). The individual molecules make the same angle with respect to the mean membrane normal regardless of their position in the "ripple" (Wack and Webb, 1989; Alecio et al., 1985; Stamatoff et al., 1982). X-Ray diffraction provides a static picture of the membrane, however, a fraction of the phospholipid molecules may well experience some angular variation, especially in the peaks and troughs of the ripples, which is averaged in the x-ray results. The motivation for examining orientational exchange in the $P_{B'}$ phase was to see if we could detect the "ripples," using a technique that is sensitive to the dynamic nature of the membrane, as a basis for observations of other membrane perturbations.

Our results clearly demonstrate a significant increase in orientational exchange in the $P_{\beta'}$ phase relative to that of the $L_{\beta'}$ phase. For $t_{mix} = 1$ ms, there is no detectable exchange over the range of 30–39°C. For a mixing time of 10 ms, there is a small amount of exchange observed at 39°C. For $t_{mix} = 50$ ms, an increase in off-diagonal intensity is observed at 34°C, the temperature of the pretransition, when the sample is heated (not shown). When cooled, the loss of off-diagonal intensity occurs between 33 and 32°C (Fig. 6). The amount of exchange is increased for $t_{mix} = 200$ ms (Fig. 7), but occurs over the same temperature range.

The question of interest is whether the observed exchange is due to either of the putative exchange mechanisms discussed above (i.e., localized lateral diffusion over the ripples or defects). The answer depends on the actual values of D_t in the $P_{\beta'}$ phase, which, as discussed above, span an enormous range in the literature. Nevertheless, some insight can be gained from considering the extreme values. From measured values of D_t, it is possible to calculate theoretical diffusion pathlengths for the values of t_{mix} used in the present study, using the relation $r = 2(D_t t_{mix})^{1/2}$ (Blackwell et al., 1987), where r is the root-mean-square displacement. Thus, if the values of Schneider et al. (1983) ($D_f = 4 \times 10^{-11}$ cm²/s, $D_s = 10^{-16}$–10^{-17} cm²/s) are correct, this would imply that the exchange is due solely to defect diffusion. Using D_f and D_s with $t_{mix} = 50$ ms, we calculate $r = 28$ and 0.04 nm, respectively; the fast diffusing component could diffuse only over a distance of one or two ripples, whereas the slow diffusing component would be immobilized on the NMR timescale. If we use the values of Kapitza et al. (1984) ($D_f = 2 \times 10^{-9}$ cm²/s, $D_s = 10^{-10}$ cm²/s), then the slow diffusing component could traverse 45 nm in 50 ms, some 2–3 ripple wavelengths, far enough for observation of the putative local exchange to be observed. The fast diffusing component could travel at least 200 nm in 50 ms (if the defect structures "channel" phospholipid along a given path, then diffusion over greater distances than those given by the above relation may occur). In order for diffusion over the liposome surface to be observed (global diffusion), the lipid would have to traverse some 1,000–2,000 nm, based on the observed exchange in the L_α phase. Thus, the FRAP results are consistent with the orientational exchange in the $P_{\beta'}$ phase originating both from local diffusion of the gel-state phospholipid over several ripples and from faster diffusion of some phospholipid in the proposed defect structures, but do not allow discrimination between these two possibilities. It appears unlikely that global diffusion would be observed.

We have not attempted to simulate the ripple-phase spectra because this would require a model for the structure of the ripples, which are not sinusoidal, and assumptions regarding the extent of excursion of the phospholipids from the bilayer normal during diffusion over the rippled surface. However, the 2D lineshape observed in the ripple phase (Fig. 6) is reminiscent of the L_α spectra of DMPC ($t_{mix} = 10$ ms) and DPPC ($t_{mix} = 1$ ms) which suggested the presence of two components of phospholipid (see Figs. 1–3); i.e., the spectra exhibit significant off-diagonal intensity and significant intensity remaining in the diagonal. This could result

from a mixture of fast and slower diffusing phospholipid, which would be consistent with orientational exchange resulting from diffusion of the faster component in the proposed defect structures.

To clarify the situation, 2D-exchange experiments were performed on oriented DPPC multibilayers in the $P_{\beta'}$ phase, oriented close to the magic angle with respect to the external magnetic field (Figs. 8 and 9). Removing the effects of membrane curvature *may* reduce or remove the exchange due to defect diffusion. This could occur if the rapid diffusion is occurring in linear defects located in the ridges and furrows of the ripples (Schneider et al., 1983). If *no* exchange was detected in the oriented gel-phase samples, this would suggest that *all* of the exchange observed in the powders was due to defect diffusion because diffusion over the ripples should not be dependent on global surface curvature.

Our results indicate an increase in off-diagonal intensity in the oriented $P_{\beta'}$ phase compared with the $L_{\beta'}$ phase, for t_{mix} = 50 ms. This is particularly clear when comparing the spectra obtained at 39°C for t_{mix} = 1 and 50 ms, where there is essentially no off-diagonal intensity for the former mixing time. This observed orientational exchange could be due to diffusion over the ripples, or to diffusion in defect structures which allow orientational exchange in oriented samples; at present we cannot differentiate between these two possibilities. However, compared to the powder spectra, where significant off-diagonal intensity is observed, the magnitude of the observed broadening in the oriented sample is relatively small. This suggests that additional reorientational mechanisms are operative in the multilamellar dispersions, the most likely possibility being the diffusion of lipids, within defect structures, over the curved surface of the liposomes. This interpretation, while not definitive, is consistent with results obtained by FRAP (Kapitza et al., 1984).

In summary, orientational exchange is observed in the $P_{\beta'}$ phase of both multilamellar dispersions and oriented multibilayers of DPPC. This exchange could originate from diffusion of phospholipids over the rippled membrane surface, and/or from diffusion within or along defect structures, with the latter process predominant in multilamellar dispersions. A more definitive interpretation of the data will require simulations of the exchange anticipated by diffusion over the ripples and in various types of defect structures. While such a treatment is beyond the scope of the present study, it is clear that 2D-orientational exchange ^{31}P NMR is one of the few NMR techniques that can provide information on the time scales of diffusive motions in the gel phases of membranes.

Orientational exchange in biological membranes

Whereas the potential of the technique for model membrane systems has been demonstrated, its practical applicability to biological membrane systems may be questioned. In this section we present preliminary studies suggesting that the 2D technique may be particularly useful in examining molecular dynamics within biological membranes. Measurements of lateral diffusion of both lipids and proteins within biological membranes by the FRAP technique has provided useful information on membrane lateral organization (Vaz et al., 1984; Tocanne et al., 1989). The ability to observe orientational exchange over the millisecond to second timescale by 2D NMR may provide complimentary information on lateral diffusion over short and long distances, and has the potential to examine localized perturbations.

One system which has been extensively characterized is the erythrocyte ghost. Human erythrocytes are biconcave cells with a diameter of 7.5 μm and a thickness of ~2 μm (Thews and Hutten, 1983). If we assume similar dimensions for the pig erythrocyte, then diffusion over the curved edges of the cell can be thought of as occurring on a sphere of radius 1 μm. From the estimated value of τ_d = 400 ms, we estimate a value for D_t of $\sim 4 \times 10^{-9}$ cm²/s at 30°C, which agrees nicely with the value of 8×10^{-9} cm²/s measured by FRAP (Vaz et al., 1984). This suggests that with further refinement, in this case greater signal-to-noise ratio and a greater number of mixing times, the 2D method will provide a means of obtaining accurate measurements of lateral diffusion coefficients of in vivo systems.

Orientational exchange experiments were also performed on intact pig spinal cord. At the outset, it should be stressed that nerve fibers have an elongated shape, and thus do not approximate spheres as do the model systems and the erythrocyte ghosts. This may have an effect on the observed exchange, and thus the conclusions below are necessarily tentative. For intact pig spinal cord, essentially no exchange is observed over the range of 1 to 20 ms, and little is observed even at t_{mix} = 200 ms. This immediately suggests that the membrane surface is relatively "smooth," i.e., any local surface perturbations are either present in small number or have little effect on the mean orientation of the phospholipid molecules with respect to the membrane normal. Nerve fibers have a diameter on the order of 1 μm. Based on the simulations, τ_d for this system is on the order of 1.3 s, which, using Eq. 6, gives $D_t = 3 \times 10^{-10}$ cm²/s. Alternatively, we can consider that diffusion through an arc of 90° can occur in a distance of 0.8 μm (1/4 the circumference). For a D_t value of 10^{-8} cm²/s, the diffusion pathlength is 0.9 μm, and for 10^{-9} cm²/s, it is still 0.3 μm,

which should give rise to observable exchange. This implies that in the pig spinal cord, lateral diffusion is much slower than 10^{-9} cm^2/s, or that the diffusion is restricted to smaller microdomains. The percolation theory of Saxton (1987, 1989) predicts that immobile obstacles, such as proteins linked to the cytoskeleton, will prevent long-range diffusion at concentrations greater than the percolation threshold; at lower concentrations, the immobile obstacles cause a reduction in the rate of lateral diffusion. The experimental support for this theory has been discussed by Vaz et al. (1984). FRAP studies on *Aplysia* neurons have been interpreted to suggest that the membrane lipids are organized into microdomains (Tocanne et al., 1989). These domains may be made up of coexisting regions of gel and liquid-crystalline lipid, or may result from protein lattices, which prevent lateral diffusion over larger areas (Tocanne et al., 1989). Evidence for these kinds of effects have been obtained from a wide variety of natural membranes (Vaz et al., 1984; Tocanne et al., 1989), and thus it is reasonable to speculate that the observed lack of exchange in the pig spinal cord is due to similar restrictions on lateral diffusion. The current results, while preliminary, demonstrate the feasibility of performing 2D-exchange experiments on biological membranes and intact tissues, and suggest that such studies may prove valuable in increasing our understanding of phospholipid diffusion and lateral organization in both biological membranes and model systems.

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REFERENCES

Alecio, M. R., A. Miller, and A. Watts. 1985. Diffraction of x-rays by rippled phosphatidylcholine bilayers. *Biochim. Biophys. Acta.* 815: 139–142.

Auger, M., and H. C. Jarrell. 1990. Elucidation of slow motions in glycoglycerolipid bilayers by two-dimensional solid-state deuteron NMR. *Chem. Phys. Lett.* 165:162–167.

Blackwell, M. F., K. Gounaris, S. J. Zara, and J. Barber. 1987. A method for estimating lateral diffusion coefficients in membranes from steady-state fluorescence quenching studies. *Biophys. J.* 51:735–744.

Bloom, M., and E. Sternin. 1987. Transverse nuclear spin relaxation in phospholipid bilayer membranes. *Biochemistry.* 26:2101–2105.

Bodenhausen, G., H. Kogler, and R. R. Ernst. 1984. Selection of coherence-transfer pathways in NMR pulse experiments. *J. Magn. Reson.* 58:370–388.

Bradshaw, J. P., M. S. Edenborough, P. J. H. Sizer, and A. Watts. 1989. A description of the phospholipid arrangement intermediate to the humidity produced L_α and H_{II} phases in dioleoylphosphatidylcholine and its modification by dioleoylphosphatidylethanolamine as studied by x-ray diffraction. *Biochim. Biophys. Acta.* 987:104–110.

Campbell, R. F., E. Meirovitch, and J. H. Freed. 1979. Slow-motional NMR line shapes for very anisotropic rotational diffusion. Phosphorus-31 NMR of phospholipids. *J. Phys. Chem.* 83:525–533.

Clayden, N. J. 1986. Observation of spin diffusion during MAS using the NOESY experiment. *J. Magn. Reson.* 68:360–362.

Connor, C., A. Naito, K. Takegoshi, and C. A. McDowell. 1985. Intermolecular spin-diffusion between ^{31}P nuclei in a single crystal of dipotassium α-D-glucose-1-phosphate dihydrate; a 1-D analogue of the 2-D exchange NMR experiment. *Chem. Phys. Lett.* 113:123–128.

Cullis, P. R., and B. de Kruijff. 1979. Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta.* 559:399–420.

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

Derzko, Z., and K. Jacobson. 1980. Comparative lateral diffusion of fluorescent lipid analogues in phospholipid multibilayers. *Biochemistry.* 19:6050–6057.

Edzes, H. T., and J. P. C. Bernards. 1984. Two-dimensional exchange NMR in static powders: interchain ^{13}C spin exchange in crystalline polyethylene. *J. Am. Chem. Soc.* 106:1515–1517.

Fahey, P. F., and W. W. Webb. 1978. Lateral diffusion in phospholipid bilayer membranes and multilamellar liquid crystals. *Biochemistry.* 17:3046–3053.

Hagemeyer, A., K. Schmidt-Rohr, and H. W. Spiess. 1989. Two-dimensional magnetic resonance experiments for studying molecular order and dynamics in static and in rotating solids. *Adv. Magn. Reson.* 13:85–130.

Jarrell, H. C., P. A. Jovall, J. B. Giziewicz, L. A. Turner, and I. C. P. Smith. 1987. Determination of conformational properties of glycolipid head groups by ^2H NMR of oriented multibilayers. *Biochemistry.* 26:1805–1811.

Jeener, J., B. H. Meier, P. Bachmann, and R. R. Ernst. 1979. Investigation of exchange processes by two-dimensional NMR spectroscopy. *J. Chem. Phys.* 71:4546–4553.

Kapitza, H. G., D. A. Rüppel, H.-J. Galla, and E. Sackmann. 1984. Lateral diffusion of lipids and glycophorin in solid phosphatidylcholine bilayers. *Biophys. J.* 45:577–587.

Larsen, D. W., J. G. Boylan, and B. R. Cole. 1987. Axially symmetric ^{31}P NMR line shapes with selective excitation in the presence of lateral diffusion on a curved surface. *J. Phys. Chem.* 91:5631–5634.

Mackay, A. L., E. E. Burnell, C. P. Nichol, G. Weeks, M. Bloom, and M. I. Valic. 1978. Effect of viscosity on the width of the methylene proton magnetic resonance line in sonicated phospholipid bilayer vesicles. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 88:97–100.

Milburn, M. P., and K. R. Jeffrey. 1987. Dynamics of the phosphate group in phospholipid bilayers. A ^{31}P nuclear relaxation time study. *Biophys. J.* 52:791–799.

Milburn, M. P., and K. R. Jeffrey. 1989. Dynamics of the phosphate group in phospholipid bilayers. A ^{31}P angular dependent nuclear spin relaxation time study. *Biophys. J.* 56:543–549.

Müller, H.-J., M. Luxnat, and H.-J. Galla. 1986. Lateral diffusion of small solutes and partition of amphipaths in defect structures of lipid bilayers. *Biochim. Biophys. Acta.* 856:283–289.

Rance, M., and R. A. Byrd. 1983. Obtaining high-fidelity spin-½ powder spectra in anisotropic media: phase-cycled Hahn echo spectroscopy. *J. Magn. Reson.* 52:221–240.

Saxton, M. J. 1987. Lateral diffusion in an archipelago: the effect of mobile obstacles. *Biophys. J.* 52:989–997.

Saxton, M. J. 1989. Lateral diffusion in an archipelago: distance dependence of the diffusion coefficient. *Biophys. J.* 56:615–622.

Schmidt, C., S. Wefing, B. Blümich, and H. W. Spiess. 1986. *Chem. Phys. Lett.* 130:84–90.

Schmidt, C., B. Blümich, and H. W. Spiess. 1988. Deuteron two-dimensional exchange NMR in solids. *J. Magn. Reson.* 79:269–290.

Schneider, M. B., W. K. Chan, and W. W. Webb. 1983. Fast diffusion along defects and corrugations in phospholipid $P_{\beta'}$ liquid crystals. *Biophys. J.* 43:157–165.

Seelig, J. 1978. ^{31}P nuclear magnetic resonance and the head group structure of phospholipids in membranes. *Biochim. Biophys. Acta.* 515:105–140.

Seelig, J., L. Tamm, L. Hymel, and S. Fleischer. 1981. Deuterium and phosphorus nuclear magnetic resonance and fluorescence depolarization studies of functional reconstituted sarcoplasmic reticulum membrane vesicles. *Biochemistry.* 20:3922–3932.

Stamatoff, J., B. Feuer, H. J. Guggenheim, G. Tellez, and T. Yamane. 1982. Amplitude of rippling in the P_{β}, phase of dipalmitoylphosphatidylcholine bilayers. *Biophys. J.* 38:217–226.

States, D. J., R. A. Haberkorn, and D. J. Ruben. 1982. A two-dimensional nuclear overhauser experiment with pure absorption phase in four quadrants. *J. Magn. Reson.* 48:286–292.

Sternin, E. 1988. Some mechanisms of transverse nuclear magnetic relaxation in model membranes. Ph.D. thesis. University of British Columbia, British Columbia, Canada. 95 pp.

Thews, G., and H. Hutton, 1983. Biophysics of respiratory gas transport. *In* Biophysics. W. Hoppe, W. Lohmann, H. Markl, and H. Ziegler, editors. Springer-Verlag, Berlin. 503–514.

Tocanne, J.-F., L. Dupou-Cézanne, A. Lopez, and J.-F. Tournier. 1989. Lipid lateral diffusion and membrane organization. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 257:10–16.

Vaz, W. L. C., Z. I. Derzko, and K. A. Jacobson. 1982. Photobleaching measurements of the lateral diffusion of lipids and proteins in artificial phospholipid bilayer membranes. *Cell Surf. Rev.* 8:83–135.

Vaz, W. L. C., F. Goodsaid-Zalduondo, and K. Jacobson. 1984. Lateral diffusion of lipids and proteins in bilayer membranes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 174:199–207.

Vaz, W. L. C., R. M. Clegg, and D. Hallmann. 1985. Translational diffusion of lipids in liquid crystalline phase phosphatidylcholine multibilayers. A comparison of experiment with theory. *Biochemistry.* 24:781–786.

Wack, D. C., and W. W. Webb. 1989. Synchrotron x-ray study of the modulated lamellar phase $P_{\beta'}$ in the lecithin-water system. *Phys. Rev. A.* 40:2712–2730.

Wefing, S., and H. W. Spiess. 1988. Two-dimensional exchange NMR of powder samples. I. Two-time distribution functions. *J. Chem. Phys.* 89:1219–1233.

Wefing, S., S. Kaufmann, and H. W. Spiess. 1988. Two-dimensional exchange NMR of powder samples. II. The dynamic evolution of two-time distribution functions. *J. Chem. Phys.* 89:1234–1244.

Wu, E.-S., K. Jacobson, and D. Papahadjopoulos. 1977. Lateral diffusion in phospholipid multibilayers measured by fluorescence recovery after photobleaching. *Biochemistry.* 16:3936–3941.