Elucidation of Cross-Relaxation Pathways in Phospholipid Vesicles Utilizing Two-Dimensional ^1H NMR Spectroscopy

Jeffrey F. Ellena, William C. Hutton,† and David S. Cafiso*

Contribution from the Department of Chemistry and Biophysics Program, University of Virginia, Charlottesville, Virginia 22901. *Received July 23, 1984*

Abstract: Two-dimensional proton cross-relaxation spectroscopy has been utilized to yield information on cross-relaxation in phospholipid vesicle systems. The rates of magnetization exchange due to cross-relaxation have been measured by examining the build-up rates of cross-peaks in the two-dimensional spectra. Spin-diffusion which is clearly indicated in these spectra occurs over limited distances and within limited domains in sonicated egg phosphatidylcholine vesicles. The extent and pattern of spin-diffusion in sonicated vesicles varies as a function of the state and type of lipid. Because of the limited rate of spin-diffusion in these systems, the development of cross-relaxation between nuclei at early mixing times can be taken as a strong indication of a close proximity between nuclei. Cross-relaxation between the hydrophobic ion tetraphenylborate and the lipid resonances of egg phosphatidylcholine vesicles has been measured and indicates that intermolecular dipolar exchange is possible in these systems. Furthermore, the development of cross-peaks from this dipolar exchange can be used to localize this ligand within the lipid matrix. Thus, two-dimensional cross-relaxation spectroscopy can provide a means to examine the conformation of lipids and localize ligands in small membrane vesicle systems.

Introduction

Nuclear magnetic resonance spectroscopy (NMR)[1] has been an invaluable tool for the study of biological membrane organization. The determination of molecular dynamics in the acyl chain region of phospholipids as well as lipid conformation has been approached with numerous NMR methods.[2] Most NMR investigations of membrane systems have been directed at measurements of the frequencies and amplitudes of molecular motion. While motion and conformation are clearly related, relatively few investigations have utilized approaches which directly measure internuclear distances in membranes.

In small molecules the measurement of nuclear Overhauser effects (NOE's) provides a measure of magnetization transfer between nuclei and can yield information on internuclear distances and conformation.[3] In membrane systems the measurement of NOE's, primarily homonuclear proton NOE's, has not been widely utilized for several reasons. Proton relaxation in membranes is dominated by strong dipolar interactions which in membrane vesicles are partially averaged by fast internal motions of the membrane lipid (e.g., rotational diffusion and alkyl chain segmental motion). A further averaging of the dipolar interactions results from a slow isotropic tumbling of the vesicles and a lateral diffusion of the lipid.[4-7] In membrane vesicles, the spacial information provided by the usual steady-state NOE experiment is lost because of strong zero quantum exchange processes which are stimulated by the slow molecular motions.[8,9] In these cases the dominance of the energy conserving transitions results in a "diffusion" of magnetization, hence a loss of spacial information.

Several methods termed "transient" and "truncated" NOE's have been developed to permit the measurement of build-up rates of the NOE in macromolecular systems. Because the build-up rates of the NOE are proportional to $1/r^6$, spacial information can be recovered,[10,11] Two-dimensional NMR methods have been developed which permit the measurement of magnetization exchange and the rates for cross-relaxation.[12,13] These have been successfully applied to macromolecular systems and provide several distinct advantages over the one-dimensional methods.[9] For example, a single two-dimensional experiment can yield a complete picture of the relaxation pathways available to various nuclei. In addition, for more complex spectra, one does not experience the selectivity problem of the one-dimensional methods encountered while irradiating crowded spectral regions. The use of low powers to overcome the selectivity problem in one-dimensional NOE methods can reduce the spacial resolution or "contrast" provided by the NOE build-up rates.[14]

In the present paper, we report the application of a two-dimensional cross-relaxation experiment for the measurement of magnetization exchange between protons in membrane vesicle systems. The rates and pattern of cross-relaxation in systems such as sonicated egg phosphatidylcholine (EPC) vesicles indicate that spin-diffusion is rather slow and limited to certain regions of the lipid molecule, As a result, the initial rates of magnetization exchange can be used as an indication of internuclear distance. Intermolecular cross-relaxation has been measured between the hydrophobic ion tetraphenylborate and lipid protons in EPC vesicles. The initial rate of this exchange is also dependent upon the close proximity of ligand and lipid protons and indicates that this technique can provide a means to localize some ligands within model membrane systems. Several other important features of the 2D cross-relaxation spectra of lipid vesicles are illustrated here. For example, unlike conventional proton T_1 relaxation rates, the diagonal peaks of these 2D spectra show a wider variation in relaxation rates, apparently reflecting the local molecular order. In addition, cross-relaxation on each side of the vesicles is revealed,

Experimental Section

Materials. Egg phosphatidylcholine (EPC) was prepared according to the procedure of Singleton et al.[15] and stored in chloroform under argon at −20 °C at a concentration of 100 mg/mL. Dipalmitoylphosphatidylcholine (DPPC), dilauroylphosphatidylcholine (DLPC),

† Present address: Monsanto Co., St. Louis, MO 63167.

(1) Abbreviations used are: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; EPC, egg phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; LOPC, dilinoleoylphosphatidylcholine; POPC, palmitoyloleoylphosphatidylcholine; DAPC, diarachidonoylphosphatidylcholine; N-Me, *N*-methyl; Ph_4B^-, tetraphenylborate; T_1, spin–lattice relaxation time.

(2) For a review, see: Jacobs, R. E.; Oldfield, E. *Prog. Nucl. Magn. Reson. Spectrosc.* **1981**, *14*, 113-136.
(3) Noggle, J. H.; Schirmer, R. E. "The Nuclear Overhauser Effect"; Academic Press: New York, 1971.
(4) Bloom, M.; Burnell, E. E.; Valic, M. I.; Weeks, G. *Chem. Phys. Lipids* **1975**, *4*, 107-112.
(5) Bloom, M.; Burnell, E. E.; MacKay, A. L.; Nichol, C. P.; Valic, M. I.; Weeks, G. *Biochemistry* **1978**, *17*, 5750-5762.
(6) Wennerström, H.; Ulmius, J. *J. Magn. Reson.* **1976**, *23*, 431-435.
(7) Bocian, D. F.; Chan, S. I. *Annu. Rev. Phys. Chem.* **1978**, *29*, 307-335.
(8) Kuroda; Y.; Kitamura, K. *J. Am. Chem. Soc.* **1984**, *106*, 1-6.
(9) Kumar, A.; Wagner, G.; Ernst, R. R.; Wüthrich, K. *J. Am. Chem. Soc.* **1981**, *103*, 3654-3658.
(10) Gordon, S. L.; Wüthrich, K. *J. Am. Chem. Soc.* **1978**, *100*, 7094-7096.
(11) Wagner, G.; Wüthrich, K. *J. Magn. Reson.* **1979**, *33*, 675-680.
(12) Jeener, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *71*, 4546-4553.
(13) Macura, S.; Ernst, R. R. *Mol. Phys.* **1980**, *41*, 95-117.
(14) Bothner-By, A. A.; Noggle, J. H. *J. Am. Chem. Soc.* **1979**, *101*, 5152-5155.
(15) Singleton, W. S.; Gray, M. S.; Brown, M. L.; White, J. L. *J. Am. Oil Chem. Soc.* **1965**, *42*, 53-56.

0002-7863/85/1507-1530$01.50/0

dioleoylphosphatidylcholine (DOPC), and dilinoleoylphosphatidylcholine (LOPC) were obtained from Sigma Chemical Co., St. Louis, MO, and used without further purification. Tetraphenylborate was obtained from Aldrich Chemical Co., Milwaukee, WI. Diarachidonoylphosphatidylcholine (DAPC) and palmitoyloleoylphosphatidylcholine (POPC) were obtained from Avanti Polar Lipids, Birmingham, AL.

Preparation of Phospholipid Vesicles. Aliquots of lipids in chloroform were dried under a stream of nitrogen and vacuum desiccated for approximately 15 h. The lipids were suspended at a concentration of 20–100 mM in a D_2O solution containing 100 mM sodium phosphate, pD 7.0, and sonicated to form vesicles as previously described.[16] The sample was centrifuged at approximately 20 000*g*'s for 15 min to remove titanium dust from the sonicator tip and float any incompletely sonicated lipid.

NMR Spectroscopy. Spectra were obtained using a Nicolet Magnetics NT-360 spectrometer equipped with a 293B pulse programmer and a 1280 data system. Standard NMC software was used to collect and process the two-dimensional data sets. The 2D cross-relaxation spectra were obtained using the following pulse sequence ($\pi/2$–t_1–$\pi/2$–$t_1/4$–$\tau_m/2$–π comp–$\tau_m/2$–$\pi/2$–t_2) where t_1, τ_m, and t_2 are the evolution, mixing, and detection periods, respectively. The incremented, composite π pulse serves to render *J* cross-peaks (including zero-quantum *J* cross-peaks) time-dependent, and symmetrization of the (F_1, F_2) data set effectively suppresses all *J* cross-peaks.[17-19] The $\pi/2$ pulses were phase-shifted in order to allow quadrature detection in both dimensions and to suppress axial-peaks using a conventional scheme common to homonuclear, two-dimensional spectroscopy.[20]

Under the experimental conditions used here, artifacts due to "t_1 noise"[20] were investigated. Samples which were physically isolated in the NMR tube were used to set detection limits for cross-peaks in the 2D cross-relaxation spectra. Under the most unfavorable conditions, t_1 artifacts gave rise to cross-peaks that had amplitudes less than 1% of their smallest corresponding diagonal peak.

Conventional, one-dimensional NOE difference spectra were obtained using both truncated[11] and transient[10] NOE methods previously described. The B_2 field which was used in these preirradiation schemes was calibrated to 27 Hz and used to selectively irradiate spins for 20 ms to 1 s.

Description of 2D Spectroscopy. Excellent descriptions of two-dimensional cross-relaxation spectroscopy have been written,[12,13] and we will point out only the salient features of this technique here. The two-dimensional spectra are a display of magnetization amplitudes in two frequency dimensions, F_1 and F_2, corresponding to the times t_1 and t_2 in the time domain. Two types of peaks are observed. Peaks on a diagonal, where $F_1 = F_2$, represent magnetization which has not been exchanged. Off-diagonal or cross-peaks arise when an exchange of magnetization has occurred between spins of different Larmor frequencies. The protons which give rise to a particular cross-peak are found by making vertical and horizontal projections back to the diagonal. Any process which induces an exchange of longitudinal magnetization serves to mix the Larmor frequencies of the spins involved. Except for water protons (HOD) which could be involved in chemical exchange, homonuclear dipole–dipole cross-relaxation is the only process which is expected to give rise to cross-peaks in the two-dimensional spectra of membrane lipids.

The integrated intensity of each diagonal or cross-peak in the 2D spectrum is proportional to a "mixing coefficient". The mixing coefficients are determined by the mixing time, τ_m, the rate constants for magnetization exchange and the rate constants for the leakage of magnetization to the lattice. In a system of spin $^1/_2$ nuclei, expressions describing the mixing coefficients have been derived.[13] For interactions between two nonequivalent nuclei, A and B, the mixing coefficients describing the cross-peak and diagonal intensities as a function of τ_m are given by:[13]

$$a_{AB} = (M_0/2R_C)(W_2^{AB} - W_0^{AB}) \exp(-R_L\tau_m)(1 - \exp(-R_C\tau_m)) \quad (1)$$

$$a_{BB} = a_{AA} = (M_0/4) \exp(-R_L\tau_m)(1 + \exp(-R_C\tau_m)) \quad (2)$$

Here, M_0 is the equilibrium magnetization, R_C and R_L are the cross-relaxation and leakage rate constants, respectively. The constants R_C and R_L are determined by the transition probabilities for double and zero quantum events, W_2^{AB} and W_0^{AB}, and the single quantum transition probability, W_1^{AB}. If AB interactions provide the only mechanism for relaxation, then R_C and R_L are given as:

$$R_C = 2|W_2^{AB} - W_0^{AB}| \quad (3)$$

$$R_L = 2W_1^{AB} + W_0^{AB} + W_2^{AB} - |W_2^{AB} - W_0^{AB}| \quad (4)$$

The "evolution" of the diagonal and cross-peaks is thus dependent upon the product of two exponentials determined by R_C and R_L. In the presence of more than one A or B spin, for example in the case of two magnetically nonequivalent methylene segments, probabilities for double and single quantum transitions among A or B spins (W_2^{AA}, W_2^{BB} or W_1^{AA}, W_1^{BB}) contribute to R_C and R_L.

Determining the transition probabilities requires a calculation of the spectral densities contributing to each transition. Clearly, an exact description of the spectral densities for the homonuclear proton cross-relaxation experiment is difficult because of the combination of both angular and internuclear distance fluctuations with molecular motion. In the present paper, our results will rely only on a qualitative interpretation of the cross-relaxation spectra. The quantitative interpretation of the data shown here and the establishment of a workable relaxation model will be discussed in a later report.

Table I. Chemical Shift Assignments and Diagonal Relaxation Times for 2D Cross-Relaxation Spectra of EPC Vesicles (25 °C)

diagonal peak (Figure 1)	group	chemical shifts (ppm) $CDCl_3$	chemical shifts (ppm) D_2O	diagonal relaxation time (ms)[b]
A	$-CH_3$	0.88	0.88	690
B	$-(CH_2)_n-$	1.26	1.29	450
C	$-CH_2-C-CO-$	1.57	1.59	140
D_1/D_2	$-CH_2-C=C-$	2.02	1.98/2.05	190/630
E	$-CH_2CO$	2.28	2.35	140
F_1/F_2	$-C=CCH_2-C=C-$	2.80	2.73/2.83	280/350
G	$-N(CH_3)_3$	3.34	3.27	350
H	$-CH_2N-$	3.75	3.72	285
I	$-CH_2OPO$ (glyceride)	3.94	4.34	200
K	CH_2OCO[a]	4.12/4.38	4.48	~60
J	$-OPOCH_2-$ (choline)	4.30	4.33	270
L	HOD		4.72	
M	CHOCO	5.18	5.21	
N	$-HC=CH-$	5.33	5.30	420

[a] The geminal protons on this glycerol segment are resolved into two distinct resonances as revealed by two-dimensional correlated spectroscopy (see text). [b] The relaxation data are best described by at least two exponentials which are determined by leakage and cross-relaxation rate constants R_L and R_C, respectively. A single exponential, nonetheless, fits well to many of the diagonal peaks, yielding the relaxation times listed here. These are intended to provide an approximate comparison of rates.

Results

Cross-Relaxation Spectra of EPC Vesicles: Diagonal Peaks. Two-dimensional cross-relaxation spectra of sonicated EPC vesicles have been obtained for a series of mixing times ranging from 50 ms to 1 s. Contour plots of four of these spectra are shown in Figure 1. Also shown in Figure 1A is a one-dimensional ^1H NMR spectrum of this lipid vesicle sample. The chemical shifts and assignments for the peaks labeled in Figure 1B are listed in Table I and are consistent with those previously given[8,21] with the exception that the C-1 glycerol protons apparently yield two distinct resonances in $CDCl_3$. As revealed by two-dimensional correlated spectroscopy, both resonances at 4.12 and 4.38 ppm are split by a strong geminal coupling. The resonance at 4.12 ppm is additionally coupled to the vicinal C-2 glycerol proton. The two C-1 resonances and the absence of coupling between the C-1 proton at 4.38 ppm and the C-2 proton suggests that this part of the glycerol backbone is locked into a configuration with a dihedral angle near 90° between the two noncoupled protons.

The relaxation of the diagonal peaks in Figure 1 will in general be characterized by at least two rate constants; however, a single

(16) Castle, J. D.; Hubbell, W. L. *Biochemistry* **1976**, *15*, 4818–4831.
(17) Macura, S.; Wüthrich, K.; Ernst, R. R. *J. Magn. Reson.* **1982**, *47*, 351–357.
(18) Baumann, R.; Wider, G.; Ernst, R. R.; Wüthrich, K. *J. Magn. Reson.* **1981**, *44*, 402–406.
(19) Wider, G.; Macura, S.; Kumar, A.; Ernst, R. R.; Wüthrich, K. *J. Magn. Reson.* **1984**, *56*, 207–234.
(20) Bax, A. "Two-Dimensional Nuclear Magnetic Resonance in Liquids"; D. Reidel: Boston, 1982.
(21) Hauser, H.; Phillips, M. C.; Levine, B. A.; Williams, R. J. P. *Eur. J. Biochem.* **1975**, *58*, 133–144.

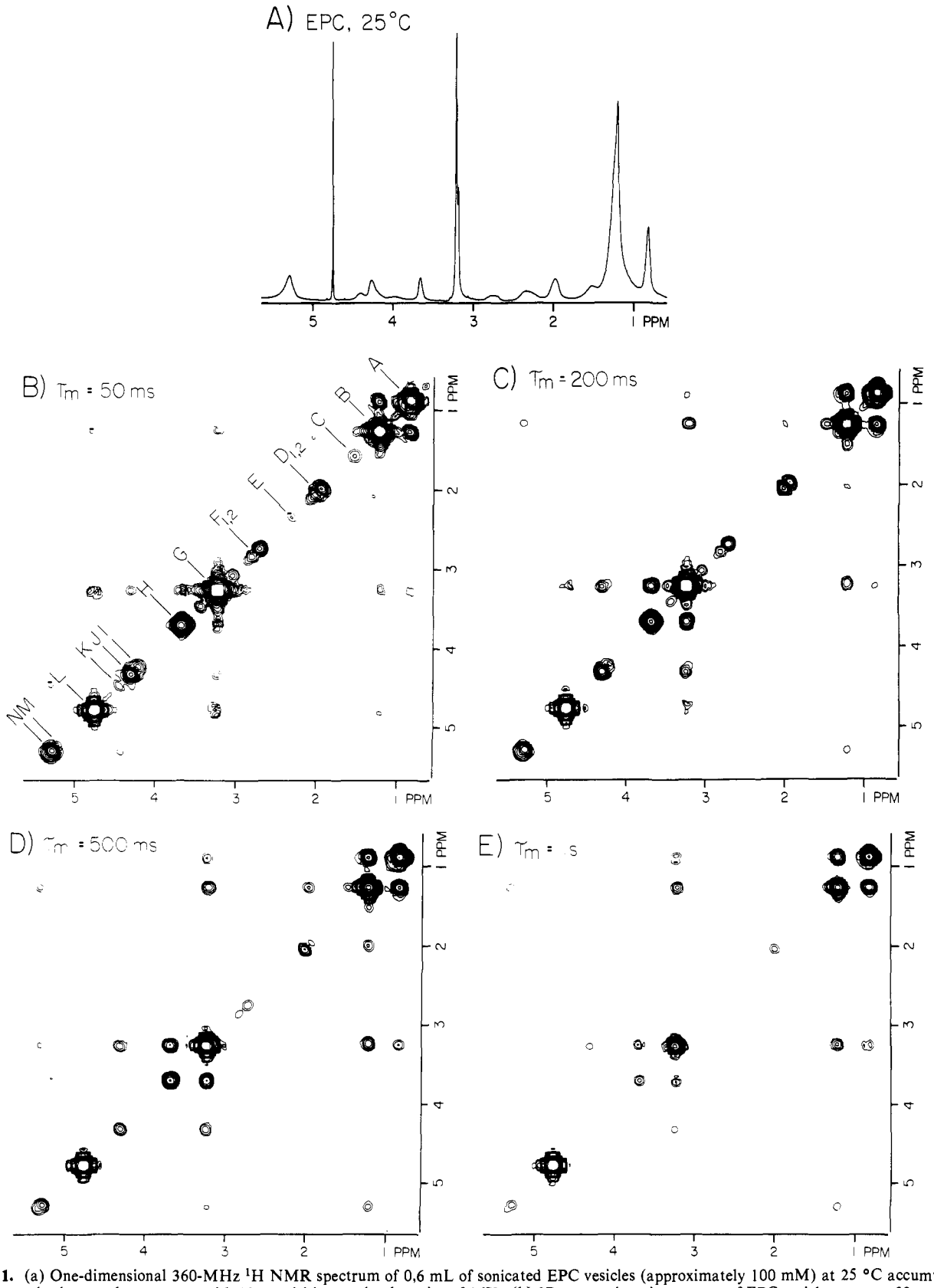


Figure 1. (a) One-dimensional 360-MHz ^{1}H NMR spectrum of 0,6 mL of sonicated EPC vesicles (approximately 100 mM) at 25 °C accumulated using a standard one-pulse sequence with 32 acquisitions and a data size of 16K. (b) 2D cross-relaxation spectra of EPC vesicles at τ_m = 50 ms. The acquisition time and data set were 49 ms and 256 data points, respectively; 128 data blocks (incremented t_1 values) were taken, each consisting of 32 scans. Each of the 14 contours plotted represents an amplitude change of $\sqrt{2}$, and the highest contour is positioned to 14% of the peak N-Me resonance. Each successive spectrum has the same scaling factor as the 50-ms plot. (c) τ_m = 200 ms. (d) τ_m = 500 ms. (e) τ_m = 1 s. The assignments for the resonances here are given in Table I.

exponential fits well to many of the diagonal evolution rates, and we have listed for comparative purposes the relaxation lifetimes for the single exponential fits to these rates (see Table I). From Figure 1 and Table I it can be seen that the fastest decaying peaks are those in or near the glycerol backbone, Peaks C, E, K, and M have virtually completely decayed by τ_m = 200 ms. As we move to the choline headgroup, the relaxation times increase, reaching a maximum at the N-Me resonance. The slowest relaxing diagonal

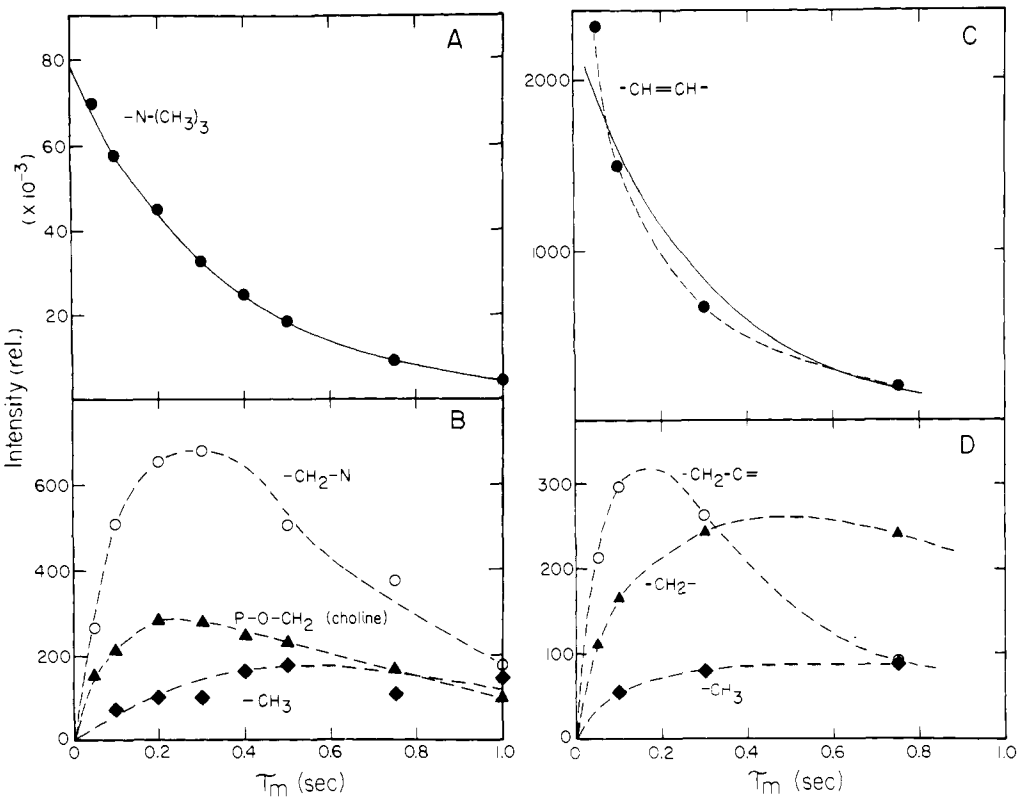


Figure 2. Amplitudes of diagonal and cross-peaks in the 2D cross-relaxation spectra of lipid vesicles as a function of the mixing time, τ_m. Solid lines (—) represent the best fit to a single exponential that can be drawn through the diagonal "evolution" curves. (a) Diagonal evolution for the N-Me (resonance G, Figure 1) in EPC vesicles. (b) Cross-peak evolution for the G, H (○), G, J (▲), and G, A (♦) resonances in EPC vesicles. (c) Diagonal evolution for the double-bond resonance (peak N) (C-9 and C-10) in DOPC vesicles. (d) Cross-peak evolution for the N, D (○), N, B (▲), and N, A (♦) resonances in DOPC vesicles (see Figure 5). The buildup of magnetization between successive groups along the alkyl chain provides strong evidence for spin-diffusion and sets limits to its rate in this lipid system.

peak excluding the solvent (HOD) is the terminal methyl resonance (A),

Several features seen on the diagonal of the two-dimensional map are not seen in the one-dimensional spectrum at 25 °C. For example, the allylic peaks labeled D and F which correspond to methylene resonances adjacent to one and two double bonds, respectively, are each resolved into two resonances split by about 0.1 ppm on the diagonal. The peak labeled D_1 relaxes approximately three times faster than the peak labeled D_2. Spectra of homogeneous lipids (dioleoylphosphatidylcholine (DOPC), dilinoleoylphosphatidylcholine (LOPC), and palmitoyloleoylphosphatidylcholine (POPC) yield single resonances for these peaks (see below), Even diarachidonoylphosphatidylcholine (DAPC) (20;4 chains) yields a single resonance for D. Since DOPC, LOPC, and DAPC yield identical chemical shifts for the peak D, it seems unlikely that the heterogeneous mixture of unsaturated lipids in EPC can account for the peaks D_1 and D_2. The ratio of peak heights is approximately 2 which suggests that the difference may be due to inside/outside packing differences in EPC (see Discussion).

Cross-Peak Evolution. At early mixing times, $\tau_m \leq 100$ ms (see Figure 1), little measurable magnetization exchange has occurred between lipid protons, In the range of 300–500 ms several strong cross-peaks develop in the 2D spectra, but the exchange is by no means complete throughout the lipid proton system and appears to be localized to certain groups of nuclei. For example, the headgroup resonances H and J efficiently cross-relax with the N-Me protons, peak G; however, only the N-Me resonance exchanges with the methylene chain, and no relaxation of the N-Me resonance with the allylic peaks F and D on the unsaturated chains can be detected (much lower contours were examined than those shown in Figure 1). Surprisingly, no exchange is seen between the two methylene segments in the headgroup (H and J). At longer mixing times (see Figure 1E), the loss of magnetization in the diagonal peaks due to leakage processes results in a decrease in the cross-peak amplitudes,

The time development (or evolution) of several diagonal and cross-peaks is shown in Figure 2, The diagonal peak for the N-Me resonance is shown in Figure 2A and decays with a characteristic lifetime of 350 ms. The cross-peaks of the N-Me protons with the remaining headgroup protons and with the terminal methyl protons are shown in Figure 2B. Significant exchange between the N-Me and terminal methyl resonances, peaks A and G, does not occur until $\tau_m \simeq 400$ ms and remains small, Also shown in Figure 3 is the diagonal peak evolution for the two resonances labeled D_1 and D_2 associated with the allylic resonances adjacent to a single double bond,

A close examination of the N-Me, methylene chain cross-peak (peaks B and G) at earlier mixing times (100 to 200 ms) clearly shows that the chemical shift of this cross-peak is displaced from the peak N-Me resonance to lower field by 0.03 to 0.05 ppm. This small difference is seen with other lipids as well, for example, in POPC and DPPC where two distinct resonances develop from peaks B and G (see Figure 5). It should be noted that a number of the cross-peaks seen at early mixing times ($\tau_m = 50$ ms) are below our "t_1-noise" limit and are likely to be artifacts of the 2D experiment. These cross-peaks do not evolve as the other cross-peaks, but simply decay with the diagonal peaks; the peaks arising from A and G, G and L, and B and L are below this limit at $\tau_m = 50$ ms. The cross-peak between the double-bond "region" and the glycerol peak, K, is well above this limit and is more than 50% the amplitude of the diagonal peak, K.

Cross-Relaxation Spectra of EPC Vesicles at 70 °C. Spectra for EPC vesicles at 70 °C with mixing times of 50 ms to 1 s have been taken (data not shown). As expected the diagonal resonances have slower relaxation rates at this temperature; in addition, several cross-peaks not seen at 25 °C are now present. In particular, exchange now occurs between the two methylene headgroup resonances (labeled H and J in Figure 1) and between the allylic resonances (D and F). The relaxation rates for the methylene resonances C and E near the carbonyl are now slow enough to permit the development of NOE's between these groups and the

Table II. Comparison of Approximate Proton Diagonal Relaxation Times for Several Lipid Vesicle Systems[a]

group	lipid (25 °C) EPC	DOPC	LOPC	DAPC	DLPC	70 °C EPC	Ph_4B^- + EPC
$-CH_3$	690	475	580	570	260	1.2 s	365
$-N(CH_3)_3$	350	300	310	280	250	820	250
CH_2-N	285	250	290	240	190	650	185
$-OPOCH_2-$ (choline)	270	185	150	160	170	550	170
$-NC{=}CH-$	417	300	340			990	270

[a] Relaxation times are given in milliseconds with the one exception as noted.

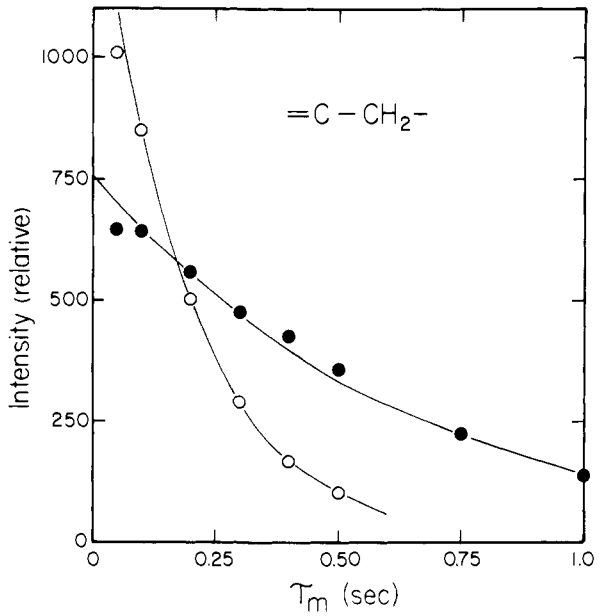


Figure 3. Diagonal peak amplitude vs. mixing time for the allylic resonance (peak D, Figure 1) in sonicated EPC vesicles. The lines (—) represent the best fit of an exponential to the two "subpeaks" seen in this resonance. Resonance D_1 (○) relaxes with a lifetime of approximately 190 ms; resonance D_2 (●) relaxes with a lifetime of 630 ms. These peaks do not appear to represent the lipid heterogeneity in EPC (see text).

methylene chain, B. A number of glycerol backbone resonances are now present, but some of the cross-peaks are difficult to assign because of the overlap with the water resonance.

One-Dimensional Transient and Truncated NOE'S. One-dimensional transient and truncated NOE experiments were utilized here to determine the "signs" of the cross-peaks shown in the 2D cross-relaxation maps, This information is lost during our 2D data processing. In all cases, both at 25 and 70 °C net positive magnetization is transferred from selectively irradiated resonances (negative NOE's) indicating that the cross-relaxation is dominated by zero-quantum (energy-conserving) transitions. To within our experimental error (approximately a 1% NOE), no positive NOE's are detected, This is in agreement with previous ^1H NOE measurements in this system.[8]

Cross-Relaxation Spectra of Homogeneous Lipids. To clarify assignments and possible pathways for cross-relaxation, we have examined several lipid systems that are in a fluid state at 25 °C. The relaxation rates for the diagonal resonances of DOPC, LOPC, DAPC, and DLPC are almost always faster than for EPC vesicles (see Table II). Again as with EPC vesicles, magnetization is restricted to certain domains, and the methylene headgroup resonances do not exchange with the alkyl chains. However, unlike EPC vesicles, the successive development of magnetization (spin diffusion) along unsaturated alkyl chains is clearly evident in DOPC, LOPC, and DAPC vesicles. The double-bond diagonal peak and allylic–methylene–terminal methyl cross-peaks with the double bond in DOPC are plotted in Figure 2C,D. The C-8 and C-11 allylic protons develop NOE's first with the adjacent double bond and the methylene chain. Eventually, this exchange is seen at the terminal methyl resonance, six methylene segments away. Thus, on this portion of the lipid molecule, a diffusion of magnetization to develop a maximal cross-peak requires 250 ms to cover six methylene segments. Strong cross-relaxation also develops between the N-Me resonance and resonances along the unsaturated chain.

In Figure 4 are shown two spectra of diarachidonoylphosphatidylcholine (DAPC) vesicles at 50 and 300 ms. At the early mixing time, cross-peaks clearly result from nuclei in close proximity; by 300 ms strong exchange along the alkyl chains is evident.

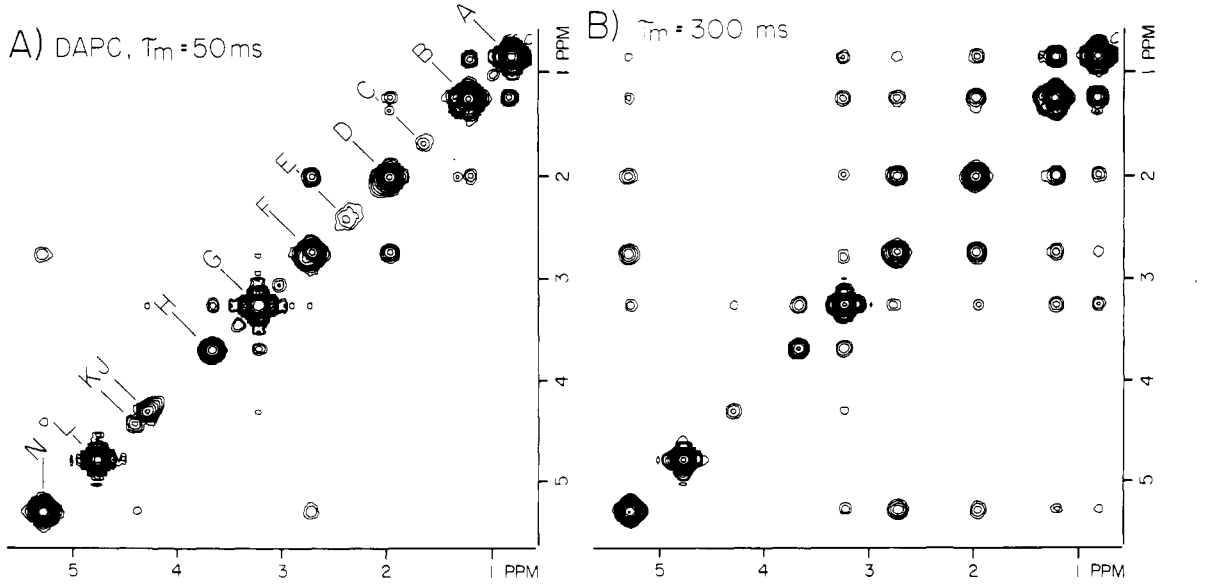


Figure 4. Two-dimensional spectra of sonicated DAPC vesicles (30 mM) for (a) τ_m = 50 ms and (b) τ_m = 300 ms, both at 25 °C. The data set was identical with that in Figure 1 except that 16 contours are plotted with the highest positioned to 30% of the amplitude of the N-Me peak in the 50-ms spectrum. At τ_m = 50 ms cross-peaks apparently provide a good indication of close internuclear distances. At τ_m = 300 ms spin-diffusion has occurred throughout the unsaturated alkyl chains (see text).

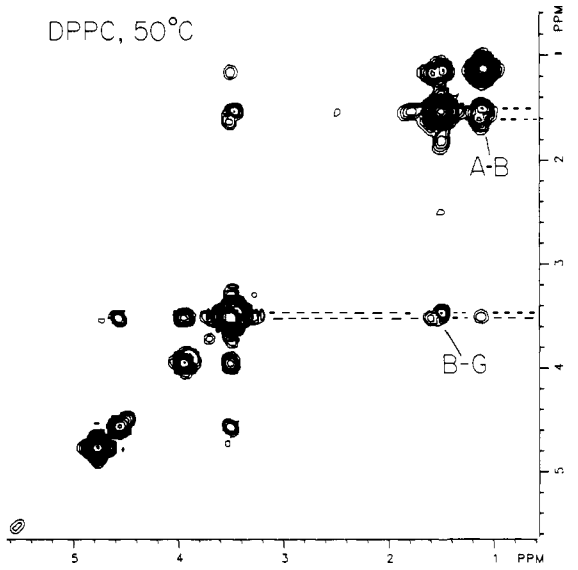


Figure 5. A 2D cross-relaxation spectrum of DPPC vesicles (100 mM) at 50 °C (τ_m = 500 ms). The data set which is 128 × 256 consists of 32 acquisitions per block. The B,G cross-peak is clearly split by the inside/outside chemical shift difference of the N-Me resonance with the cross-relaxation developing more rapidly and intensely on the inner vesicle surface (see text). There are also two A,B cross-peaks indicating two populations of terminal methyl–methylene chain interactions.

In Figure 5 is shown the 2D map for DPPC. Cross-relaxation between the N-Me resonance and the methylene chains results in two distinct cross-peaks which are shifted by the inside/outside N-Me splitting seen in the one-dimensional ^{1}H NMR spectrum. In DPPC two distinct cross-peaks also develop between the terminal methyl and methylene chain.

Cross-Relaxation Spectra of Vesicles and Hydrophobic Ions. The interaction of the tetraphenylborate anion (Ph_4B^-) with phospholipid vesicles was studied to determine the extent of intermolecular cross-relaxation between ligands such as Ph_4B^- and the vesicle system and to establish criteria for utilizing 2D cross-relaxation techniques to localize these types of ligands within the membrane structure. Shown in Figure 6A–C are 2D contour maps at three different mixing times, 50–300 ms, for egg PC containing 20 mol % Ph_4B^-. The hydrophobic ion lipid mixture was cosonicated and produced a uniform vesicle phase which was identical in appearance with pure EPC vesicles. As previously described, phenyl ring-current shifts are induced in the lipid resonances due to the hydrophobic ion,[22] Here we see shifts of approximately 0.2 ppm induced in the headgroup resonances G and H. The relaxation rates of the lipid diagonal resonances are faster, and the cross-relaxation is much more efficient than for pure EPC (see Table II).

At early mixing times, τ_m = 50 ms (Figure 6A), cross-peaks between the Ph_4B^- phenyl resonances (particularly the meta resonance) and the N-Me headgroup resonance are seen. As the mixing time is lengthened, intermolecular exchange develops with the other remaining headgroup resonance, J, and with the methylene and terminal methyl protons. At τ_m = 300 ms there is virtually complete cross-relaxation seen among the lipid and hydrophobic ion resonances.

Discussion

Our original objective in carrying out the present cross-relaxation study of model membrane systems was to determine whether useful information on internuclear separation in membranes could be obtained using the 2D cross-relaxation technique. There are relatively few NMR techniques capable of yielding this information in membrane systems. We have been particularly interested in how ligands such as hydrophobic ions interact with membranes. A better understanding of hydrophobic ion binding

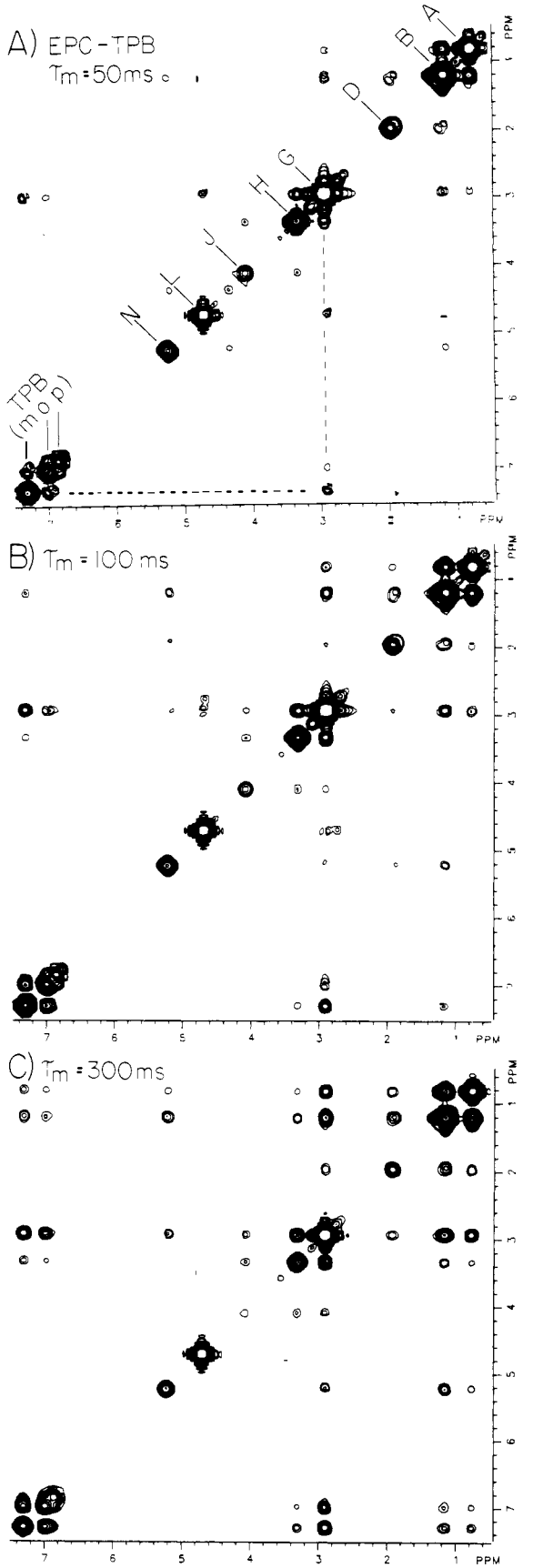


Figure 6. The 2D cross-relaxation spectra of sonicated EPC vesicles (100 mM) containing 20 mol % Ph_4B^- at 25 °C. Fourteen contours are plotted in these maps with the highest contour positioned to 10% of the amplitude of the N-Me peak in the τ_m = 50 ms spectrum. Each 128 × 256 spectrum consisted of 32 acquisitions with an acquisition time of 46 ms. The relaxation times are shorter and the spin-diffusion is greatly enhanced compared with EPC vesicles (Figure 1) at 25 °C. At τ_m = 50 ms (a) and τ_m = 100 ms (b), strong TPB, lipid cross-peaks provide a good indication of close proximity as indicated by phenyl ring-current shifts (see text). For (c) τ_m = 300 ms (and longer), efficient spin-diffusion obscures the useful structural information contained in the 2D spectra.

(22) Levine, B. A.; Sackett, J.; Williams, R. J. P. *Biochim. Biophys. Acta* **1979**, *550*, 201–211.

could greatly improve our ability to interpret membrane electrostatic measurements made with these ions.[23]

Spin-Diffusion Rates in Membranes. In membrane vesicles the interactions leading to magnetization transfer between protons are predominantly energy-conserving (zero-quantum) transitions resulting in negative NOE's (or positive cross-peaks in the 2D cross-relaxation spectra). Under these conditions a diffusion of magnetization along groups of closely associated protons, "spin-diffusion", is possible. Spin-diffusion is clearly revealed in the cross-relaxation spectra of phospholipid vesicles; see, for example, the Ph_4B^-/EPC or DAPC 2D spectra (Figures 6 and 4 shown above). Surprisingly, in almost all cases, spin-diffusion does not completely "couple" all the nuclei of the lipid matrix as measured by this 2D technique. Magnetization exchange over longer distances generally takes a moderate time to develop, depending upon the region of lipid and the "dynamic" state of the membrane. In DOPC bilayers, a maximal exchange of magnetization along the alkyl chain between the C-12 and C-18 segments requires approximately 30 ms/$-CH_2-$. In EPC, spin-diffusion along the unsaturated chain is much slower than in DOPC, LOPC, or DAPC vesicles, and no significant magnetization exchange in EPC is detected between the terminal methyl group and the allylic protons. These differences are likely due to differences in the local molecular ordering of the membrane lipid. The efficiency of spin-diffusion in these vesicle systems is expected to vary with the amplitude of the internal lipid motion (i.e., with a dipolar order parameter, S_{HH}). More efficient spin-diffusion as a result of stronger zero-quantum interactions should occur when the order parameter is larger. Rates of spin-diffusion have been previously utilized in this way to examine lipid packing in vesicles.[24]

Proton T_1 measurements in lipid vesicles (as measured by conventional methods) do not show a wide variation in relaxation rates presumably because cross-relaxation among the nuclei tends to average out the relaxation rates. In the 2D spectra of vesicles there is much more significant difference in the diagonal relaxation rates presumably because magnetization that is transfered between nuclei appears in the cross-peaks and is not included in the diagonal magnetization.[25]

Headgroup, Alkyl-Chain Cross-Relaxation. As shown above, the N-Me resonance can exchange with resonances on the phospholipid alkyl chains. In EPC vesicles the N-Me resonance eventually exchanges with the terminal methyl resonance (peaks A and G in Figure 1), reaching a maximum over a period of approximately 400–500 ms. Since no significant spin-diffusion down the unsaturated chain is occurring, this exchange is either facilitated by spin-diffusion down the saturated (sn-1) alkyl chain in EPC or by a close proximity of some N-Me, terminal methyl groups. These possibilities are currently being tested.

In our EPC 2D cross-relaxation spectra at early mixing times, the N-Me methylene chain ($-CH_2-$) cross-peaks which develop are shifted from the peak diagonal resonance by approximately 0.04 ppm. This is about the chemical-shift difference expected for the internal shifted N-Me resonance. In POPC, two separate cross-peaks clearly develop which are shifted by exactly the internal/external N-Me splitting (data not shown). In DPPC vesicles (Figure 6) this same splitting is also seen, and two distinct cross-peaks develop between the headgroup and chains. While the shift seen here is small, the result has been highly reproducible in these lipids, strongly suggesting that the upfield N-Me/$-CH_2-$ cross-peak is dominated by interactions on the internal monolayer of EPC, DOPC, and DPPC vesicles, Cross-relaxation is more efficient for the upfield peak, consistent with the higher lipid packing density and greater order parameter expected on the internal surface of small sonicated vesicles.[26,27]

Heterogeneity in EPC Unsaturated Chain Resonances. In the 2D spectra of EPC vesicles, methylene peaks adjacent to double bonds are composed of two distinct peaks (D_1, D_2 in Figure 1). One surprising observation is the factor of 3 difference in relaxation rates for the two peaks. Because PC from egg contains a mixture of unsaturated chains (i.e., 18:1, 18:2, 20:4, 22:6), the peaks D_1 and D_2 might result from the heterogeneity of unsaturated chains. In chloroform, however, resonance D (Figure 1) is a single peak despite the heterogeneity of EPC. In DOPC, LOPC, and DAPC vesicles, all peaks from these methylene resonances have approximately the same chemical shift. Also, the ratio between the amplitude of the two peaks is approximately 2:1 (D_1/D_2) which would be hard to reconcile with the percentages of 20:4 or 22:6 in EPC (ca. 4 and 1%, respectively). A likely possibility to consider is that the difference is due to a packing asymmetry between the inside and outside monolayers of EPC vesicles (the surface area ratios are approximately 2:1). This conclusion would be consistent with the relaxation data, since the alkyl chain on the inner monolayer (especially nearer the bilayer center) is expected to have a lower order parameter than the outer chain (hence, a slower relaxation).[27] Since DOPC, LOPC, and DAPC yield only a single resonance, this conclusion would also imply that the packing asymmetry splitting this peak is peculiar to EPC vesicles. Clearly there are other possibilities, and any two phospholipid configurations in slow exchange could explain these results. For example, a domain separation of EPC lipids in these small vesicle systems could account for these results.

Cross-Relaxation in the Phospholipid Headgroup. Of the three resonances in the headgroup, only the N-Me resonance cross-relaxes with the alkyl chain protons. Spin-diffusion does not dominate relaxation among the choline resonances as indicated by the lack of cross-relaxation between the two methylene segments (peaks H and J). The lack of exchange here is surprising, because of their close proximity, and is likely the result of an equivalence between magnetization exchange via single and double quantum transitions. Note that if a single critical correlation time[13] characterized the relaxation between these methylene protons, heating the sample should recover the NOE between these groups and result in a net nonconservative magnetization exchange (positive NOE). While heating the sample does result in an H–J cross-peak, the NOE between these protons at 70 °C is negative.

It is important to note that the development of a cross-peak between resonances H and J at 70 °C is not simply the result of the decrease in the diagonal relaxation rates at this temperature. The cross-relaxation rate between H and J as with the allylic resonances F and D has increased at 70 °C. A quantitative treatment and interpretation of the cross-relaxation seen among the headgroup and allylic resonances will be presented later.

Binding of Tetraphenylborate at Bilayers. Our original goal in producing 2D cross-relaxation spectra of lipid vesicles was to determine how useful this spectroscopy would be for examining proximity between added ligands (such as hydrophobic ions) and the membrane lipid. Clearly, the rate and extent of spin-diffusion will limit the spacial resolution of this technique. Tetraphenylborate is a hydrophobic ion that is thought to bind strongly to the lipid interface,[22] and we have examined the binding of Ph_4B^- using 2D cross-relaxation spectroscopy. As shown above (Figure 6), cross-relaxation between the phenyl protons and lipid occurs at early mixing times and is accompanied (as previously shown)[22] by phenyl ring current shifts. Thus, a close approach of Ph_4B^- and the N-Me protons is indicated both by ring shifts and strong cross-relaxation at early mixing times. The concentrations of Ph_4B^- used here are large to clearly indicate the ring current shifts (we have been able to work at Ph_4B^-/lipid ratios 20 times less).

The presence of spin-diffusion with Ph_4B^- is readily apparent and is greatly enhanced over that normally seen in EPC bilayers. By 500 ms we have achieved strong cross-relaxation among almost

(23) Cafiso, D. S.; Hubbell, W. L. *Annu. Rev. Biophys. Bioeng.* **1981**,*10*, 217–244.

(24) Schuh, J. R.; Utpal, B.; Müller, L.; Chan, S. I. *Biochim Biophys. Acta* **1982**, *687*, 219–225.

(25) Because of the possibility of numerous intra- and intermolecular dipolar interactions, the 2D-diagonal relaxation rates will clearly be much more difficult to interpret in terms of local molecular ordering than ^{13}C or ^{2}H T_1 relaxation rates.

(26) Huang, C.; Mason, J. T. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 308–310.

(27) Dill, K. A.; Flory, P. J. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 676–680.

all lipid protons. This is presumed to result from a change in lipid dynamics and not a chemical exchange of Ph_4B^-. EPR experiments reveal an increase in the order parameter of both headgroup and chain-labeled lipids with high concentrations of Ph_4B^-,[28] and higher concentrations of Ph_4B^- can fuse neutral EPC bilayers. While the time scale examined by EPR is much shorter than that for NMR, a decrease in the amplitudes of motion with Ph_4B^- is consistent with previous observations and the enhanced rate of spin-diffusion seen here.

The Ph_4B^- data presented here clearly indicate that intermolecular exchange is possible in membrane systems and that exchange measured with 2D spectroscopy can provide information on binding. Not all "ligands" added to membrane systems cross-relax with lipid protons; halothane, a general anesthetic, reveals no anesthetic–lipid cross-peaks even at high concentrations (data not presented). The difference between this compound and Ph_4B^- presumably lies in the strength and specificity of the Ph_4B^- binding.

Conclusions

Two-dimensional cross-relaxation ^1H NMR can be utilized to reveal cross-relaxation pathways in phospholipid vesicle systems. Cross-relaxation among lipid protons in fluid-phase vesicles (such as sonicated egg yolk lecitin) develops over modestly long time periods (hundreds of milliseconds) and is apparently restricted to a few relaxation networks. As a result, spin-diffusion which does occur in this system does not totally obscure the useful structural information which can be obtained from the rates of magnetization exchange. Strong, rapidly developing cross-relaxation provides good evidence for a close proximity of nuclei as demonstrated by the tetraphenylborate–lipid cross-peaks. Several other noteworthy features of the 2D lipid spectra have been observed. Unlike conventional proton T_1 measurements, the diagonal peaks on these 2D spectra evolve with rates that are not averaged by cross-relaxation processes. Thus, the diagonal relaxation rates should reflect the local molecular dynamics and ordering. Finally, in several lipid systems asymmetry between the inside and outside surfaces of small sonicated vesicles is revealed in headgroup–alkyl chain cross-relaxation. Such cross-relaxation may provide further insight into the packing of lipids in small vesicle systems.

Acknowledgment. This research was supported by NSF grant BNS 8302840 and a Dreyfus Foundation grant for young faculty in chemistry (both to D.S.C.). We thank Drs. Carl Trindle and Michael F. Brown for helpful discussions during the course of this work,

Registry No. DOPC, 10015-85-7; LOPC, 6542-05-8; DAPC, 17688-29-8; DLPC, 18285-71-7; DPPC, 2644-64-6; Ph_4B^-, 4358-26-3.

(28) Seidah, N. G.; Roy, G.; Laprade, R.; Cyr, T.; Dugas, H. *Can. J. Biochem.* **1976**, *54*, 327–335.

Chemical Applications of the Two-Dimensional $^2H,^{13}C$ NMR Shift Correlation Experiment

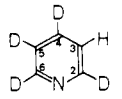
Joachim R. Wesener and Harald Günther*

Contribution from the Fachbereich 8, OC II, University of Siegen, Postfach 101240, D-5900 Siegen, Germany. *Received August 7, 1984*

Abstract: Deuterium-labeling studies in mechanistic organic and bioorganic chemistry rely on the analysis of deuterated carbon sites in organic molecules. From the various techniques provided for this purpose by modern high-resolution pulse NMR spectroscopy, the two-dimensional $^2H,^{13}C$ shift correlation experiment is of particular interest. It combines the selectivity of polarization transfer with signal dispersion in two frequency dimensions. Promising analytical and mechanistic applications of this new technique are presented, and its scope is evaluated. A critical comparison of the available ^{13}C NMR pulse methods for deuterium-labeling studies and their relevant hardware requirements are given.

An important aspect of mechanistic studies in organic and bioorganic chemistry based on deuterated compounds is the analysis of deuterated carbon sites. In this respect, modern pulse NMR[1] provides a number of useful techniques. Simple spin-echo spectroscopy with modulation of transverse ^{13}C magnetization by $^{13}C,^1H$ and/or $^{13}C,^2H$ spin–spin coupling, for example, allows signals of nondeuterated carbons (C, CH, CH_2, CH_3), partially deuterated carbons (CHD, CHD_2, CH_2D), and fully deuterated carbons (CD, CD_2, CD_3) to be characterized and thus yields information about the number of protons and/or deuterons attached to a particular site.[2,3] For mixtures of deuterated and nondeuterated material, $\{^2H\}^{13}C$ polarization transfer experiments are especially helpful, because they act like a filter eliminating the resonances of nondeuterated carbons.[4,5] The spectra then only contain the signals of partially and fully deuterated groups.

In practice, due to pulse and phase imperfections in the polarization transfer experiment, the latter result is obtained only if the signal intensities of the nondeuterated positions are not dramatically different from those of the deuterated ones. For mixtures containing an excess of nondeuterated material, the signals for CH, CH_2, and CH_3 groups usually cannot be completely suppressed, which complicates, or even prevents, the interpretation of the resulting spectra. This fact is demonstrated by a polarization transfer experiment for a mixture of 55 mg of pyridine-*2,4,5,6-d*$_4$ (**1**) and 3 g of isotope-free pyridine (**2**), where the signals of the deuterated carbons can only be assigned with the help of their isotope effects (Figure 1).



1

We found that problems of this kind can most easily be solved with the recently described two-dimensional $^2H,^{13}C$ shift corre-

(1) For a review, see: Benn, R.; Günther, H. *Angew. Chem.* **1983**, *95*, 381; *Angew. Chem., Int. Ed. Engl.* **1983**, *22*, 350.
(2) Wesener, J. R.; Günther, H. *Org. Magn. Reson.* **1983**, *21*, 433.
(3) Wesener, J. R.; Schmitt, P.; Günther, H. *J. Am. Chem. Soc.* **1984**, *106*, 10.
(4) Rinaldi, P. L.; Baldwin, N. J. *J. Am. Chem. Soc.* **1983**, *105*, 7523.
(5) Bendall, M. R.; Pegg, D. T.; Wesener, J. R.; Günther, H. *J. Magn. Reson.* **1984**, *59*, 223.

0002-7863/85/1507-1537$01.50/0