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^{13}C Nuclear Magnetic Resonance Studies of Egg Phosphatidylcholine

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Summary. Spin lattice relaxation times (T_1) and apparent spin-spin relaxation times (T_2^*) derived from linewidth have been used to investigate model membranes composed of egg yolk phosphatidylcholine. T_1 measurements appear to be largely dominated by segmental motion and as a consequence are not very sensitive to small changes in membrane structure. On the contrary, apparent T_2^* times are shown to be sensitive to such changes in the membrane and are thus suggested as a useful tool for further investigation of membrane structure.

Of all the techniques used to study the dynamic properties of model membranes, ^{13}C NMR may be the most formidable. Advances in instrumentation, such as Fourier transform techniques, give the investigator an opportunity to simultaneously inspect numerous reporting probes from different regions in the molecule without perturbing the local environment as in the case with ESR probes. As a prelude to natural membrane work, we have studied the dynamic properties of egg yolk phosphatidylcholine in various physical states. While previous investigators have emphasized ^{13}C spin lattice relaxation times (T_1) as an indication of molecular motion [15, 19], it is proposed that apparent spin-spin relaxation times (T_2^*) are far more sensitive to subtle changes in membrane structure.

Materials and Methods

Phosphatidylcholine was isolated from egg yolk using the method of Singleton and co-workers [25]. Thin-layer chromatography of the product using the solvent system of $CHCl_3/MeOH/H_2O$ (65:24:4) revealed only a single spot in an iodine atmosphere.

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Salts and solvents employed were of the highest purity available. Water was glass-distilled, degassed, and saturated with nitrogen.

In the course of the studies described below, several different preparations of phosphatidylcholine (PC) were employed: hydrated PC, PC in methanol, sonicated and unsonicated PC vesicles. The hydrated material was prepared by equilibration of PC, previously dried *in vacuo* at 78 °C for 12 hr, with water in a desiccator. The course of the hydration process was followed by monitoring the weight gain of the PC sample as a function of time of equilibration; 24 hr was required to complete the equilibration process, at which time the sample contained 23 per cent water on a weight basis. Unsonicated dispersions of PC in water were prepared by dissolving 0.8 g of PC in chloroform in a 50-ml round bottom flask. This solution was then taken to dryness on a rotary evaporator under a nitrogen atmosphere. To the flask containing the PC film was added 4 ml of an aqueous solution containing 0.1 M KCl and 5 mM tris-(hydroxymethyl) aminomethane (Tris), pH 8.0. The flask was then agitated gently by hand until no PC remained adhering to the walls. The thick white suspension so obtained was transferred to a 13-mm NMR tube and layered with argon. This type of preparation is referred to as unsonicated liposomes.

Sonicated dispersions of PC in water, termed sonicated liposomes, were prepared by transferring a sample of the unsonicated liposomes to a 20-ml test tube followed by sonication for 2 hr under a continuous stream of nitrogen in a Branson sonicator equipped with a steep horn tip and operating at power level six. During the sonication, the sample was immersed in an ice bath; at the conclusion of the process, the sample temperature was 13 °C. The sonicated liposomes were subsequently transferred to a 13-mm NMR tube and layered with argon. Phosphatidylcholine content of the samples employed was determined on the basis of phosphorous content [18].

The equipment and conditions used to obtain the spectra have been described previously [2, 12]. T_1 values were obtained via $180° - \tau - 90°$ pulse sequence, where τ is the waiting period between the 180° pulse and the 90° pulse. Details of the procedure of getting T_1 values from the data have been described elsewhere [1, 2, 27]. T_1 values are estimated to be accurate to $\pm 10\%$. T_2^* values were estimated from linewidths using the formula $T_2^* = \frac{1}{\pi \nu_{\frac{1}{2}}}$ where $\nu_{\frac{1}{2}}$ is the width of peaks in Hz at one half the height. T_2^* values are estimated to be accurate to $\pm 40\%$.

Results

Egg yolk phosphatidylcholine is a mixture of molecules with a variety of pairs of acyl chains. A typical distribution is given in Table 1. Despite the complexity of egg yolk phosphatidylcholine, it is possible to discuss the ^{13}C NMR resonances of the various carbon atoms in terms of a typical phosphatidylcholine molecule.

Assignments of the various resonances have been based on model compounds that resemble certain combinations of atoms in egg yolk phosphatidylcholine [21]. These results are presented in Table 2. Assignments agree with those suggested by others [8, 15, 19].

The major concern of this work has been to determine the usefulness to T_1 and T_2^* relaxation measurements in natural abundance ^{13}C NMR studies

Table 1. Fatty acid composition of egg phosphatidylcholine [a]

Type	% Total	% at 1 [b]	% at 2 [c]
16:0	37.1	70.7	1.7
16:1	1.4	1.7	0.9
18:0	11.6	22.2	2.0
18:1	34.9	5.5	68.7
18:2 & 18:3	12.0	0.9	21.8
20:4	3.0	–	4.9

[a] From data of Tattrie *et al.* [26].
[b] Esterified at carbon atom 1 of the glycerol backbone.
[c] Esterified at carbon atom 2 of the glycerol backbone.

of membrane structure. T_1 measurements have usually been the method of choice compared to the more technically difficult T_2 measurements. However, one can get an approximate idea of T_2 times from linewidth measurements. While such an apparent T_2 (i.e. T_2^*) may include contributions from other factors like magnetic field inhomogeneity, chemical exchange, and chemical shift nonequivalence, it nevertheless may offer some insight into membrane structure as well as a comparison to T_1 times. Each of the above-mentioned factors can be minimized with regard to phospholipid vesicles so that T_2^* is a reflection of the rotational motions that contribute to spin-spin relaxation. Of the three factors, instrumental broadening due to magnetic field inhomogeneity may be the most critical. Broadening due to magnetic field inhomogeneity is negligible (less than 1.0 Hz) in all cases except that of egg yolk phosphatidylcholine dissolved in methanol, where the resonances are extremely sharp (Fig. 1). To avoid any chance of instrumental broadening affecting the linewidths, an arbitrary lower limit of 8 Hz (or T_2^* of 40 msec) has been adopted, though many measured resonances of the phosphatidylcholine molecule in methanol are less than this lower limit. Therefore, any T_2^* times less than 40 msec (or linewidths greater than 8 Hz) can be considered reliable with regard to instrumental broadening. Second, chemical exchange can also cause an increased broadening of linewidths that is unrelated to the true dynamics of rotation. In a pure phospholipid vesicle system this change can only be from one side of the bilayer to the other, assuming that all sites in the lateral plane of the bilayer are equivalent. Kornberg and McConnell have shown that the half time for such a process is in the order of hours [13], far too slow to be the cause of any significant broadening of the resonances. Finally, the effects of chemical

Table 2. Chemical shift[a] and assignments of ^{13}C resonances of egg yolk phosphatidylcholine

Functional group[b]	Phosphatidylcholine in methanol[c]	Sonicated[d] vesicles	Unsonicated[e] vesicles	Hydrated[f]
$\underset{\sim}{C}=O$	19.1 (1) 19.4 (1)	19.1 (1)	19.2 (1)	20.8 (1)
$-CH_2=\underset{\sim}{C}H-CH_2-CH_2$	62.3 (2)	62.6 (2)	62.4 (2)	64.5 (2)
$-CH=\underset{\sim}{C}H-CH_2-CH=CH-$	64.0 (3)	64.2 (3)	64.2 (3)	65.5 (3)
Glycerol $H-\underset{\sim}{C}-O$ (2)	121.7 (4)	121.6 (3a)		
Choline $-\underset{\sim}{C}H_2-\overset{\oplus}{N}$	126.0 (5)	124.5 (4)	126.6 (4)	128.8 (4)
Glycerol $H_2-\underset{\sim}{C}-O-\overset{O^{\ominus}}{\overset{\|}{\underset{\|}{P}}}$ (3) $\underset{O}{}$	126.5 (6)			
		128.9 (5)		
Glycerol H_2-C-O (1)	129.1 (7)			
Choline $\underset{\sim}{C}H_2-CH_2-\overset{\oplus}{N}$	129.9 (8)	132.9 (6)	133.1 (5)	
Choline $\overset{\oplus}{N}-(CH_3)_3$	139.9 (9)	138.3 (7)	138.4 (6)	139.6 (5)
MeOH	144.4 (10)			
$-\overset{O}{\overset{\|}{C}}-\underset{\sim}{C}H_2-$	159.2 (11)	159.0 (8)		
$-\underset{\sim}{C}H_2-CH_2-CH_3$	161.2 (12)	160.3 (9)		
Main $(\underset{\sim}{C}H_2)$	163.2 (13)	163.2 (10)	162.5 (7)	162.5 (6)
$\underset{\sim}{C}H_2-CH=CH-$	166.0 (14)	166.1 (11)	166.0 (8)	165.0 (7)
$-CH=CH-\underset{\sim}{C}H_2-CH=CH-$	167.3 (15)	166.8 (12)	168.2 (9)	168.0 (8)
$-\overset{O}{\overset{\|}{C}}-CH_2-\underset{\sim}{C}H_2$	168.2 (16)			
$\underset{\sim}{C}H_2-CH_3$	170.5 (17)	170.5 (13)	170.2 (10)	170.0 (9)
$-\underset{\sim}{C}H_3$	179.4 (18)	179.4 (14)	179.4 (11)	179.0 (10)

[a] Chemical shifts are upfield from CS_2.
[b] The carbon atom that is underlined with $\sim$ represents the carbon atom in question. The numbering of the glycerol carbon in parentheses refers to its position on the glycerol backbone. Unless otherwise noted, the assignment refers to the fatty acid acyl chain.
[c–f] The numbers in parentheses are the peaks designated in Figs. 1–4.

shift nonequivalence must be contended with. Egg yolk phosphatidylcholine dissolved in methanol should be fairly isotropic and one might expect that chemical shift nonequivalence has very little importance in the calculation of T_2^*. Once an anisotropic bilayer is formed, however, the situation be-

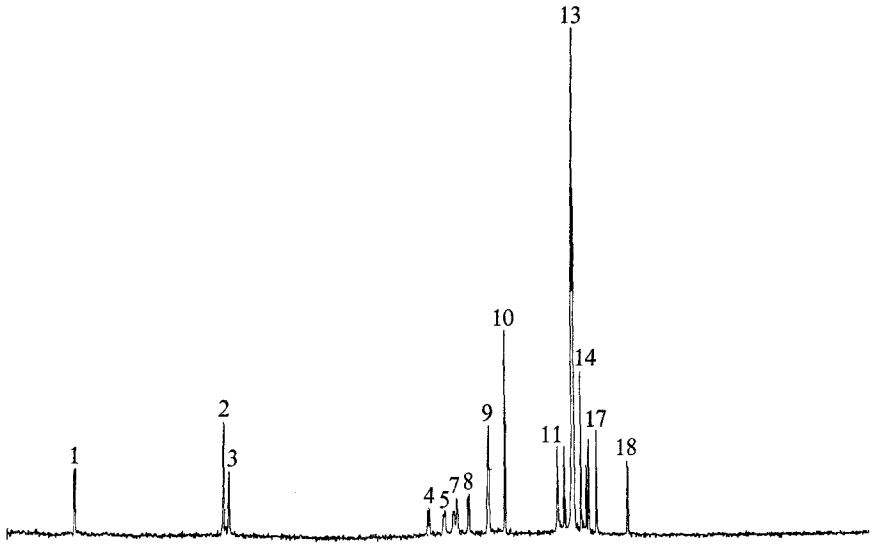

Fig. 1. Proton decoupled natural abundance 13-carbon Fourier transform spectrum of 0.94 M egg yolk phosphatidylcholine in methanol. The spectrum was recorded following 512 scans, with recycle time of 2.7 sec. The range of the spectrum is 0 to 250 ppm upfield from CS_2

comes more complex. With sonicated vesicles, the small radius of curvature may cause the phosphatidylcholine molecules in the inner and outer halves of the bilayer to be packed differently and, therefore, have different magnetic environments. Such differences could be reflected in slight changes in chemical shift that would cause broadening of the linewidth unrelated to rotational dynamics. Any possible chemical shift nonequivalence would be greatest in the polar head group regions, decrease for carbon atoms closer to the center of the bilayer; since then the difference of the radius of curvature between the two halves of the bilayer is becoming less. By the use of chemical shift reagents, the inner and outer choline peaks can be separated. Proton NMR studies indicate that any possible chemical shift nonequivalence with sonicated vesicles is minimal, especially at the magnetic field strength use in this study [4, 16]. The lack of chemical shift nonequivalence at the polar head group where the differences between the inner and outer halves should be expected to be the greatest, would indicate that chemical shift non-equivalence is negligible as a cause of broadening in sonicated phospholipid systems.

Finally, there is experimental evidence from proton NMR that T_2^* is a faithful probe for motion in vesicle systems. Lee *et al.* [14] have studied sonicated egg phosphatidylcholine vesicles under various conditions. They came to the conclusion that linewidth measurements are a poor choice for

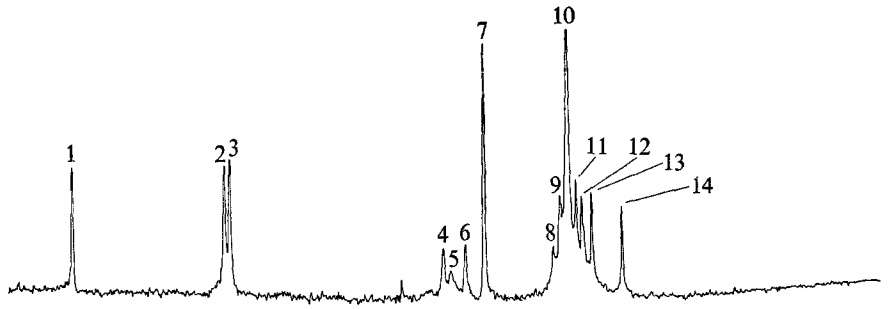

Fig. 2. Proton decoupled natural abundance 13-carbon Fourier transform spectrum of sonicated liposomes (0.35 M). The spectrum was recorded following 8192 scans, with a recycle time of 2.7 sec. The range of the spectrum is 0 to 250 ppm upfield from CS_2

investigating rotational motion in phospholipid vesicles since the T_1 times were always longer than the T_2^* measurements from linewidths. However, Horowitz *et al.* [9] using spin-echo techniques were able to get true T_2 times for various resonances of sonicated egg phosphatidylcholine vesicles. Using the relation $\nu_{\frac{1}{2}} = 1/\pi T_2$ and from the value of Horowitz *et al.* [9] of the spin echo T_2 time of the $-\overset{+}{\mathrm{N}}-(CH_3)_3$ group at 20 °C, one gets a linewidth of 4.2 Hz. This is the same width as seen by Lee *et al.* [14] for the same system. The $-\overset{+}{\mathrm{N}}-(CH_3)_3$ resonance is the only resonance that can be directly compared; however, it would appear to indicate that in phospholipid vesicle systems T_2^* is a reliable indicator of T_2 relaxation behavior.

In total it appears that for the egg phosphatidylcholine systems studied here, the complicating factors of instrumental broadening, chemical exchange, and chemical shift nonequivalence do not make large contributions to the T_2^* times. Hence, it seems that one can use T_2^* times as an indicator of dynamic rotational motion, especially in comparison to T_1 times.

The question still remains whether ^{13}C NMR does indeed give some insight into the structure of model membranes. The comparison of T_1 and T_2^* times for various different structures of egg yolk phosphatidylcholine should indicate the extent of structural changes that can be detected by both parameters.

Egg yolk phosphatidylcholine when dissolved in methanol (Fig. 1) is probably in the form of small aggregates [5] but relatively isotropic compared to the bilayer systems. Yet both T_1 and T_2^* times indicate that a phosphatidylcholine molecule in methanol has a gradient of rotational motions along the acyl chain. This type of behavior has been seen with other aggregated systems [6, 28].

In contrast to the egg phosphatidylcholine in methanol, sonicated egg phosphatidylcholine in vesicles (Fig. 2) forms an anisotropic system. We

Table 3. ^{13}C spin-lattice relaxation times of egg yolk phosphatidylcholine

Functional group[a]	PC in MeOH	Sonicated vesicle	Unsonicated vesicle	Hydrated
	T_1 (sec)	T_1 (sec)	T_1 (sec)	T_1 (sec)
$-\underset{\sim}{C}=O$	2.23	2.56	—	—
$-\underset{\sim}{C}H=CH-CH_2$	0.748	0.72	0.634	0.342
$CH=\underset{\sim}{C}H-CH_2-\underset{\sim}{C}H=CH-$	1.253	1.04	1.09	0.393
Glycerol $H-\underset{\sim}{C}-O$ (2)	0.142	—	—	—
Choline $\underset{\sim}{C}H_2-\overset{\oplus}{N}$	0.300	0.289	0.216	0.119
Glycerol $H_2-\underset{\sim}{C}-O-P(O^{\ominus})=O$ (3)	0.112	—	—	—
Glycerol $H_2-\underset{\sim}{C}-O$ (1)	0.072	0.072	—	—
Choline $\underset{\sim}{C}H_2-CH_2-\overset{\oplus}{N}$	0.279	0.34	—	—
Choline $\overset{\oplus}{N}-(\underset{\sim}{C}H_3)_3$	0.55	0.61	0.514	0.362
$-C(=O)-\underset{\sim}{C}H_2-$	0.240	0.27	—	—
$-\underset{\sim}{C}H_2-CH_2-CH_3$	0.658	0.77	—	—
Main $(\underset{\sim}{C}H_2)$	0.489	0.53	0.45	0.296
$\underset{\sim}{C}H_2-CH=CH-$	0.616	0.54	0.48	0.254
$-CH=CH-\underset{\sim}{C}H_2-CH=CH-$	0.785	0.44	0.46	—
$-C(=O)-CH_2-\underset{\sim}{C}H_2$	0.254	—	—	—
$\underset{\sim}{C}H_2-CH_3$	2.19	1.29	1.36	
$-\underset{\sim}{C}H_3$	3.11	3.81	3.31	2.9

[a] The carbon atom that is underlined with ~ represents the carbon atom in question. The numbering of the glycerol carbons in parentheses refers to their position on the glycerol backbone. Unless otherwise noted, the assignment refers to the fatty acid acyl chain.

can make some prediction of what should happen in this system in comparison to phosphatidylcholine dissolved in methanol especially in terms of which areas of the molecule will become more immobilized on formation of the bilayer. The choline head group should be relatively unaffected in terms of rotational behavior because of its extension from the surface. The glycerol region should be highly immobilized in the vesicle as compared to phosphatidylcholine in methanol. This is accounted for by the necessity of the glycerol backbone to be anchored at the surface. Finally, the acyl carbon

should be relatively immobilized near the surface but should be in a nearly isotropic condition near the end of the acyl chain. We would therefore expect as the carbon atoms become more restricted in their motion, that their rates of relaxation should decrease. Inspection of Table 3 shows that T_1 values do not seem to conform to the above predictions. The only significant difference in T_1 time between phosphatidylcholine dissolved in methanol and sonicated vesicles is that the penultimate carbon atom on the acyl chain is slightly immobilized in the sonicated vesicle. Other than that resonance, all other T_1 times of phosphatidylcholine in both physical states are within experimental error of each other. The similarity in T_1 times from two very different structures is a surprising result. However, if T_2^* times for sonicated vesicles are used (Table 4), then numerous differences between the sonicated bilayer vesicle and phosphatidylcholine dissolved in methanol are seen. The choline head retains its mobility, the glycerol backbone is somewhat immobilized as are the main acyl carbons (e.g., the carbon atoms 4–13 on the acyl chain), and the carbon atoms near the end of the acyl chain retain their fluidity compared to egg yolk phosphatidylcholine in methanol. Unlike the T_1 measurements, the T_2^* do reflect the assumed structural changes that take place in going from phosphatidylcholine in methanol to a sonicated vesicle.

The physical differences between sonicated and unsonicated vesicles are small but significant. These differences are manifested in such diverse physical properties as density [24] and osmotic sensitivity [11]. From geometrical considerations alone the structures of the two vesicles should be different [24]. As before, one can predict what types of changes should be seen upon going from sonicated to unsonicated vesicles. From ESR studies it is known that the acyl chain region of sonicated vesicles is more disordered than that of large unsonicated vesicles [17]. ^{1}H NMR has emphasized the same conclusion [24]. Dielectric dispersion studies have shown that the dielectric constant of the sonicated vesicle interior is much higher than might be expected, perhaps due to water being intercalated in the bilayer [20]. Ultracentrifuge studies indicate that in homogenous sonicated vesicles there is an appreciable amount of bound water at the interface [10]. Finally, ESR measurements have demonstrated that the external polar head groups have more freedom than the internal polar head groups in sonicated vesicles [13]. All of this information points to definite differences between the sonicated and unsonicated vesicles. The glycerol backbone should be packed closer together in the unsonicated vesicles, decreasing the amount of water intercalated at the bilayer interface. The carbon atoms near the center of the acyl chain should be less mobile in the unsonicated vesicle than in the

Table 4. ^{13}C T_2^* relaxation times of egg yolk phosphatidylcholine

Functional group[a]	Phosphatidylcholine in methanol T_2 (msec)	Sonicated vesicles T_2 (msec)	Unsonicated vesicles T_2 (msec)	Hydrated phosphatidylcholine T_2 (msec)
$-\underset{\sim}{C}=O$	>40	30.7	7.7	5.7
$-\underset{\sim}{C}H=CH-CH_2$	>40	24.2	12.8	8.5
$CH=\underset{\sim}{C}H-CH_2-CH=CH-$	>40	24.2	17.1	10.2
Glycerol $H-\underset{\sim}{C}-O$ (2)	26.7			
Choline $\underset{\sim}{C}H_2-\overset{\oplus}{N}$	22.8	17.8	11.0	4.9
Glycerol $H_2-\underset{\sim}{C}-O-P(O^{\ominus})=O$ (3)	26.7			
		12.1		
Glycerol $H_2-\underset{\sim}{C}-O$ (1)	32.0			
Choline $\underset{\sim}{C}H_2-\underset{\sim}{C}H_2-\overset{\oplus}{N}$	32.0	21.1	11.8	5.3
Choline $\overset{\oplus}{N}-(\underset{\sim}{C}H_3)_3$	32.0	33.8	25.7	13.9
$-C(=O)-\underset{\sim}{C}H_2-$	>40	16.9		
$-\underset{\sim}{C}H_2-CH_2-CH_3$	>40	13.0		
Main $(\underset{\sim}{C}H_2)$	26.6	13.5	3.5	2.8
$\underset{\sim}{C}H_2-CH=CH-$	>40	14.1	11.0	
$-CH=CH-\underset{\sim}{C}H_2-CH=CH-$	>40	14.1	8.5	
$-C(=O)-CH_2-\underset{\sim}{C}H_2$	>40			
$\underset{\sim}{C}H_2-CH_3$	40	28.2	11.0	
$-\underset{\sim}{C}H_3$	40	37.6	26.7	9.0

[a] The carbon atom that is underlined with ~ represents the carbon atom in question. The numbering of the glycerol carbons in parentheses refers to their position on the glycerol backbone. Unless otherwise noted, the assignment refers to the fatty acid acyl chain.

sonicated vesicle. The choline head group should be only slightly less mobile if at all, and the acyl chain should still have considerable fluidity near its terminal point.

If ^{13}C NMR is to have any extensive use in studying membrane phenomenon, it should be able to detect differences between sonicated and unsonicated vesicles (Figs. 2 and 3). An inspection of Table 2 reveals a rather disturbing result: there are no differences in the T_1 values of the sonicated and unsonicated vesicle. All the T_1 times are within experimental error, and

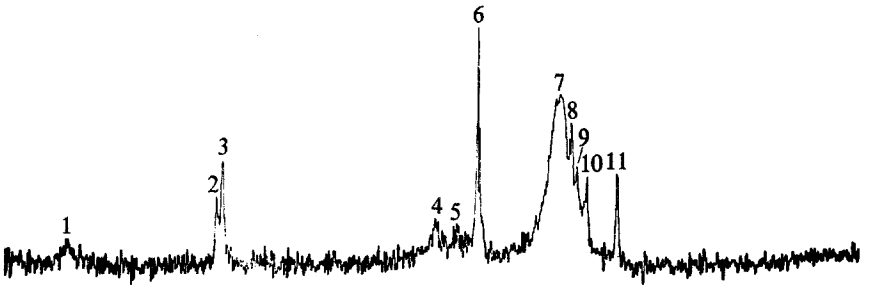

Fig. 3. Proton decoupled natural abundance 13-carbon Fourier transform spectrum of unsonicated liposomes of egg yolk PC (0.34 M). The spectrum was recorded following 2048 scans, with a recycle time of 2.7 sec. The range of the spectrum is 0 to 250 ppm upfield from CS_2

in addition the T_1 times for the unsonicated vesicles are very close to those of phosphatidylcholine dissolved in methanol. This similarity of T_1 times for the unsonicated vesicles has also been reported previously [7, 19]. T_2^* times, on the other hand (Table 4), do conform to our expectations for the differences between sonicated and unsonicated vesicles. The choline methyl groups of the unsonicated vesicle are slightly less mobile but within experimental error of the choline methyl groups of the sonicated vesicle. However, noticeable differences appear at the glycerol backbone. The glycerol carbons are so broadened that they disappear into the background noise level indicating a great increase in T_2^* of the glycerol carbons. The carbonyl groups likewise are quite reduced in mobility. However, the most amazing aspect is the high degree of immobilization of the main acyl carbon atom (carbon atoms that are relatively near the interface of the bilayer). A 4-fold decrease in the mobility is seen; this type of behavior correlates well with ESR studies of such vesicles [17]. The carbon atoms near the double bonds in the acyl chain are not nearly as affected probably due to localized disorder caused by the double bond. Finally, as might be expected, the carbon atoms near the end of the acyl chain are slightly inhibited compared to the sonicated vesicle, but not nearly to the extent seen with the atoms near the interface. It would seem that T_2^* is, therefore, a better probe of the small but significant structural changes seen in sonicated as compared to unsonicated vesicles.

To ascertain whether or not such changes in T_2^* and the lack of change in T_1 are artifacts, yet another sample of phosphatidylcholine was investigated: the hydrate sample (Fig. 4). Such a sample should be highly ordered compared to unsonicated vesicles. One might expect that such a sample is highly immobilized at the polar head group (due to lack of water). Also the glycerol region and acyl carbon atoms should be more ordered. In

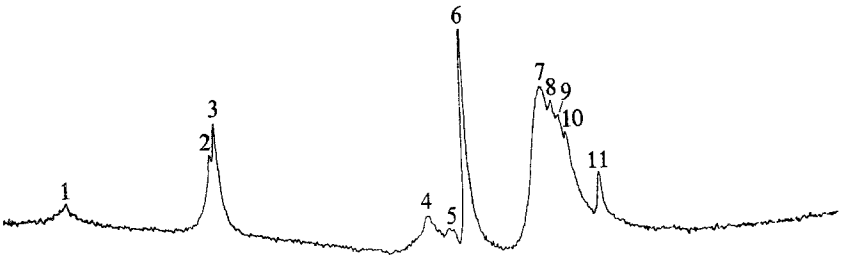

Fig. 4. Proton decoupled natural abundance 13-carbon Fourier transform spectrum of 23% hydrated egg yolk phosphatidylcholine. The spectrum was recorded following 10,496 scans, with a recycle time of 1.25 sec. The range of the spectrum is 0 to 250 ppm upfield from CS_2

this case, the T_1 values (Table 3) do reflect these expectations. The T_1 values indicate that the choline head group is immobilized, and that throughout the entire acyl chain various atoms are less mobile than in the case of the vesicles. Surprisingly, the terminal methyl group of the acyl chain remains relatively mobile according to the T_1 data. The T_2^* values in Table 4 indicate the same decreases in the mobility of the carbon atoms in the hydrated sample compared to the unsonicated vesicles. The polar head group motions are decreased, but the acyl carbons are not much less mobile than those of unsonicated vesicles. However, there is a drastic decrease in the mobility of the terminal methyl group of the acyl chain compared to the unsonicated vesicles.

Discussion

The basic question that arises from this study is why T_1 times are relatively insensitive to large structural changes in the phospholipid vesicle and conversely why do T_2^* times reflect the same structural changes. The answer probably lies in the anisotropic nature of the bilayer. In an anisotropic system, the restricted motion of the molecule prevents it from rotation in all angles in space. Unlike isotropic systems in which a single rotational correlation time τ_c can describe the rotational behavior, the anisotropic system requires two rotational correlation times $\tau_\perp$ and $\tau_\parallel$, to describe its motions. Motions parallel to the axis orientation would be characterized by $\tau_\parallel$, while those motions perpendicular to the axis of orientation would be characterized by $\tau_\perp$. Using these two types of rotational motions, the effect of the structure of the bilayer on both T_1 and T_2^* times can be demonstrated.

Allerhand and co-workers have shown in anisotropic systems such as polystyrene polymers [3] and trimethyl ammonium bromide alkyl micelles [28]

that the T_1 times are dominated by segmental motion. These segmental motions can be represented by the rotational correlation time $\tau_{\parallel}$. The physical forces that cause micelle formation are similar to those that contribute to the formation of the bilayer. As a consequence, the T_1 times in the phospholipid bilayer should also be dominated by segmental motion. Even the motion of phosphatidylcholine in methanol is somewhat anisotropic due to the small aggregated micelles [5]. The similarity of T_1 times of phosphatidylcholine in methanol, sonicated and unsonicated vesicles can therefore be best explained by the similarity of segmental motions in all three systems. Only in the case of the hydrated sample do the T_1 times show significant differences. The decrease in T_1 times would indicate that the segmental motions of the phosphatidylcholine in the hydrated sample have become more restricted. Unfortunately, the spectrometer used in this study had no provision for temperature control. However, a recent study using phospholipids enriched with ^{13}C in the acyl chains reported that the T_1 times enriched carbons of both the sonicated and unsonicated vesicles did increase with temperature [7]. The activation energy calculated for the T_1 times of the various carbon atoms was similar to that expected from segmental motion along the acyl chain.

T_2^* times, on the other hand, measure different types of molecular motions. As demonstrated by Seiter and Chan using stochastic theory [23], in a bilayer the T_2 times should be dominated by $\tau_{\perp}$. These off-axis rotational motions are very sensitive to structure in the bilayer. As the structure of the vesicle becomes more ordered, the large amplitude rotational motions characterized by $\tau_{\perp}$ become more restricted and more anisotropic. Apparently rather small changes in the membrane structure are manifested in rather large changes in $\tau_{\perp}$ and consequently T_2^*.

It is, however, possible that the differences in T_2^* values might arise from chemical shift nonequivalence. Thus, the broader line of the unsonicated vesicles spectrum could be an envelope of slightly different, but unresolvable chemically shifted resonances. It does not, however, seem likely that this is the case. Since all phospholipid molecules in the system are chemically identical, differences in chemical shift must arise from differences in molecular packing density. Differences in molecular packing must, in turn, be due to differences in bilayer curvature. However, in large unsonicated vesicles the majority of the phospholipid is in concentric lamellae of essentially infinite radii of curvature. On the basis of purely geometrical considerations, Sheetz and Chan have shown that differences in curvature become negligible when the bilayer radius is larger than about 800 Å [24]. In contrast to this situation, the effect of bilayer curvature is

maximal in the sonicated vesicles and thus chemical shift nonequivalence should be greatest in these structures. Although proton NMR studies have demonstrated a chemical shift nonequivalence between phosphatidylcholine N-methyl protons on the inner and outer bilayer faces in single-walled vesicles of minimum radius [16], no such shift is seen in the ^{13}C NMR resonance [22]. Since no chemical shift nonequivalence is observed in the spectrum of ^{13}C N-methyl carbons under the favorable conditions existing in the small single-walled vesicles, it seems unlikely that shift nonequivalence can make a substantial contribution to the width under the unfavorable conditions existing in unsonicated vesicles. It thus seems reasonable that the differences in the ^{13}C T_2^* times obtained in the bilayer systems do in fact reflect differences in molecular organization.

Needless to say, further work is needed to confirm and extend these initial findings in interpretation of T_1 and T_2^* times in phospholipid vesicles. Work is presently being pursued in the direction of using ^{13}C-enriched phospholipids [7, 22]. Experimental evidence indicates that T_1 and T_2^* relaxation times are apparently measuring two different rotational motions in phospholipid bilayers. T_1 times are dominated by segmental motions; T_2^* times reflect mainly the slower, large amplitude motions within the bilayer. These motions that dominate T_2^* are very sensitive to molecular structure of the vesicle. As a consequence, T_2^* or T_2 spin echo may offer a greater insight into small changes in membrane structure. However, T_1 times will contain a great deal of information concerning the effect of certain agents, such as cholesterol and proteins, on the segmental motions of the phospholipid molecule. Future membrane studies using both T_1 and T_2^* measurements should offer more information about the total state of membrane lipids than either relaxation time can do alone.

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