^{13}C NMR Studies on Bilayers Formed from Synthetic di-10-methyl-stearoyl phosphatidylcholine Enriched with ^{13}C in the N-methyl Carbons

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Received August 6, 1974

SUMMARY

^{13}C NMR relaxation measurements have been carried out on phospholipid bilayer systems formed from synthetic di-10-methyl-stearoyl phosphatidylcholine with 92% enrichment in one of the N-methyl carbons. Studies on single-walled vesicles prepared by sonication from this lipid, and on large multi-lamellar liposomes show that although T_1 values are nearly the same, T_2^* values are markedly different. It is proposed that equivalent segmental motions in the two systems give rise to similar T_1 values. The T_2^* values, on the other hand, are consistent with the view that the single-walled vesicles have a more disordered molecular organization than do the multi-lamellar bilayers.

INTRODUCTION

Most current concepts of biological membrane structure are based on the premise that a major portion of the lipid component is present in bilayer form and as such constitutes a barrier matrix for the organization of protein components (1). This central position of the lipid bilayer in biological membrane structure has focused attention on the structure and properties of simple bilayer systems. Much information has been obtained from fluorescent and ESR probes, and from proton NMR studies of lamellar lipid systems (2,3,4).

Recently ^{13}C NMR has been used to obtain information about molecular organization in phospholipid bilayers (5). In principle information can be obtained about each carbon atom in the molecule, although the low natural abundance of the ^{13}C nucleus combined with its smaller magnetic moment relative to hydrogen requires long term signal averaging to obtain useful results. In addition, the resonances from individual atoms cannot always be resolved. These limitations can be overcome by selective enrichment of specific atoms by chemical synthesis.

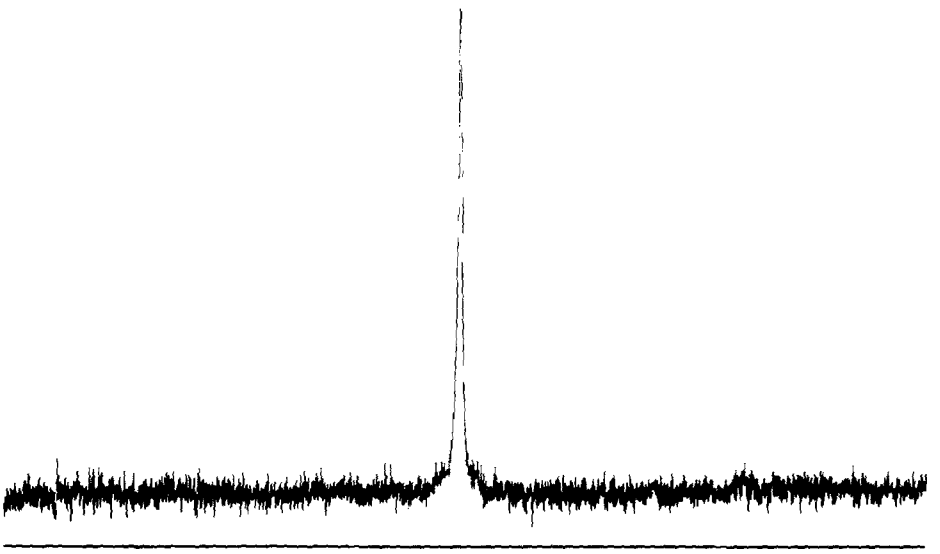

Fig. 1. Normal Fourier transformed spectrum of sonicated vesicles. Concentration 0.05 M; 1000 scans; recycle time 2.1 sec. No exponential filter was used in the transformation of the spectrum.

As the first step in a systematic NMR study of specifically enriched ^{13}C phospholipids, we report here preliminary experiments on synthetic di-10-methyl-stearoyl phosphatidylcholine with 92% ^{13}C enrichment of one of the N-methyl carbons of the choline moiety. Attention is directed to determination of the spin-lattice relaxation time, T_1, and the apparent spin-spin relaxation time, T_2^*, for small single-walled liposomes and for large multi-lamellar liposomes. Previous studies have shown that the packing density of phospholipid molecules in the bilayer of the single-walled vesicle is less than in the multi-lamellar bilayers (4,6,7). The ^{13}C NMR results reported herewith show that T_2^* values for the two systems are markedly different although T_1 values are similar. It is proposed that equivalent segmental motions in the two systems give rise to the similar T_1 values. The T_2^* values, on the other hand, are consistent with the view that the single-walled vesicles have a more disordered molecular organization than do the multi-lamellar bilayers.

EXPERIMENTAL

The synthesis of 10-methyl-stearic acid will be discussed in greater detail in a forthcoming publication (8). In brief, the ethyl ester of 10-keto stearic acid was synthesized according to the method of Hubbell and McConnell (3). The conversion of the 10-keto stearic ester to the 10-methyl-stearic acid was accomplished using the Wittig reaction as described by Chasin and Perkins (9).

The fatty acid was then incorporated into phosphatidylcholine following established procedures (10). The ^{13}C labeled choline was prepared by dissolving a slight excess of redistilled N-dimethyl ethanolamine in dry benzene. $^{13}CH_3I$ (92.3 mole %) was then added. The stoppered reaction flask was held for two days at room temperature. The ^{13}C choline iodide was removed from the reaction by filtration and then washed with dry benzene. Choline iodide was converted to the acetate salt by passage through an anion exchange column (IR-45) previously equilibrated with acetate.

The di-10-methyl-stearoyl phosphatidylcholine was converted to the corresponding phosphatidic acid using the method of Dawson (11). Thin layer chromatography indicated no residual phosphatidylcholine. ^{13}C labeled phosphatidylcholine was then prepared from the phosphatidic acid by condensation of the ^{13}C choline acetate with the di-10-methyl phosphatidic acid according to Aneja and Chandra (12). The yield based on the phosphatidic acid was 42% (136μmoles).

Large, multi-lamellar vesicles were prepared as follows: 1 ml of 0.1 M KCl, 10 mM Tris (pD 8.0) was added to 50 μmoles of phospholipid lyophilized overnight. The dispersion was then vortexed for 1 min and stored at 4°. Small, single-walled vesicles were prepared by adding 3 ml of the buffer to the lyophilized material. The resulting dispersion was then sonicated to optical clarity using a Branson sonicator at 4°. Undispersed lipid and titanium fragments were removed by cen-

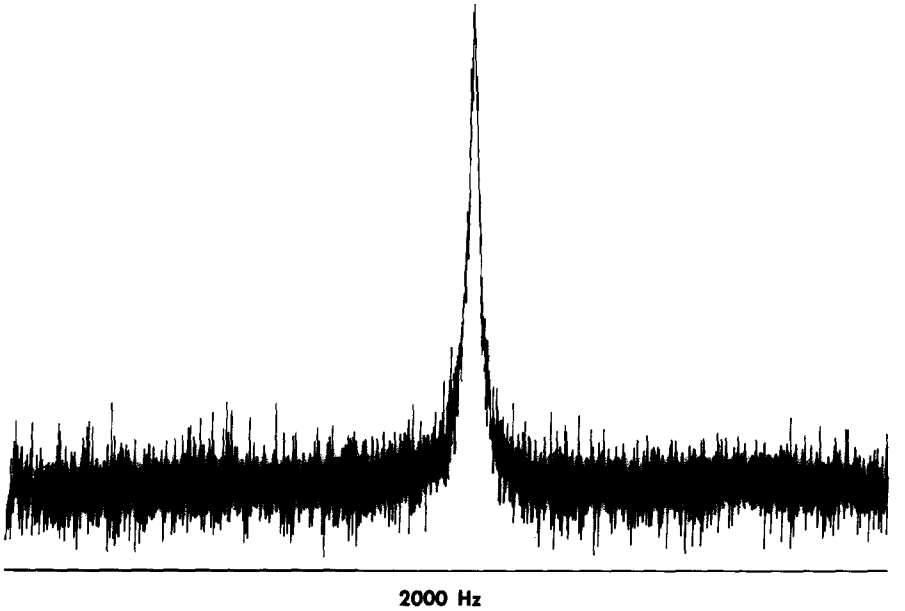

Fig. 2. Normal Fourier transformed spectrum of unsonicated vesicles. Concentration 0.05 M; 1000 scans; recycle time 1.2 sec. No exponential filter was used in the transformation of the spectrum.

trifugation at 10,000 X g for 10 min. All samples for NMR work were prepared in D_2O (98.8%).

Spectra were obtained with a JEOL-PRT-100 spectrometer at 23°. T_2^* times were determined using the relation $T_2^* = 1/\pi\Delta\nu_{\frac{1}{2}}$ where $\Delta\nu_{\frac{1}{2}}$ is the width in Hz of the peak at half height. The line shapes were assumed to be Lorentzian. Widths were determined after 1000 scans in which the recycle time was at least 5 T_1's. No exponential filter was used in transforming the spectra. T_1 measurements were determined with the usual precautions (13), using the standard 180^0-τ-90^0 pulse sequence in which the recycle time was greater than 5 T_1's. Exponential filtering was used in order to increase the signal to noise ratio. T_1 times were evaluated from the relation $A = A_0 [1 - 2 \exp(\tau/T_1)]$, where A_0 is the equilibrium amplitude of the peak and A is the amplitude after the waiting period, τ, between the 180^0 pulse and the 90^0 pulse (14). Precision of T_1 and T_2^* is estimated to be $\pm$ 15%.

D_2O (98.8%) and $^{13}CH_3I$ were obtained from Bio Rad. All other chemicals were of reagent grade.

RESULTS AND DISCUSSION

The synthetic phospholipid, di-10-methyl-stearoyl phosphatidylcholine has a liquid crystalline-crystalline phase transition below 0^0 (15). In many respects its physical properties are similar to egg phosphatidylcholine. However, unlike this natural product, it contains no double bonds and is therefore stable to oxidation. In addition the methyl branch may be enriched with ^{13}C and positioned at known points along the acyl chains. Work utilizing this family of ^{13}C derivatives is now in progress.

The spectra of the single-walled vesicles and the unsonicated multilamellar vesicles are shown in Figures 1 and 2, respectively. Relaxation times are given in Table I. It is apparent that although the T_1 times of both the sonicated and unsonicated vesicles are the same, there is a greater than 2-fold difference in the T_2^* times. Similar differences in the line width have been noted previously (16,17). The T_1 measurements are in excellent agreement with data of Levine et al (5). The semilog plots of the amplitude versus τ are

Table I

	T_1 (msec)	T_2^* (msec)
single-walled vesicles	270	27
multi-lamellar liposomes	260	11

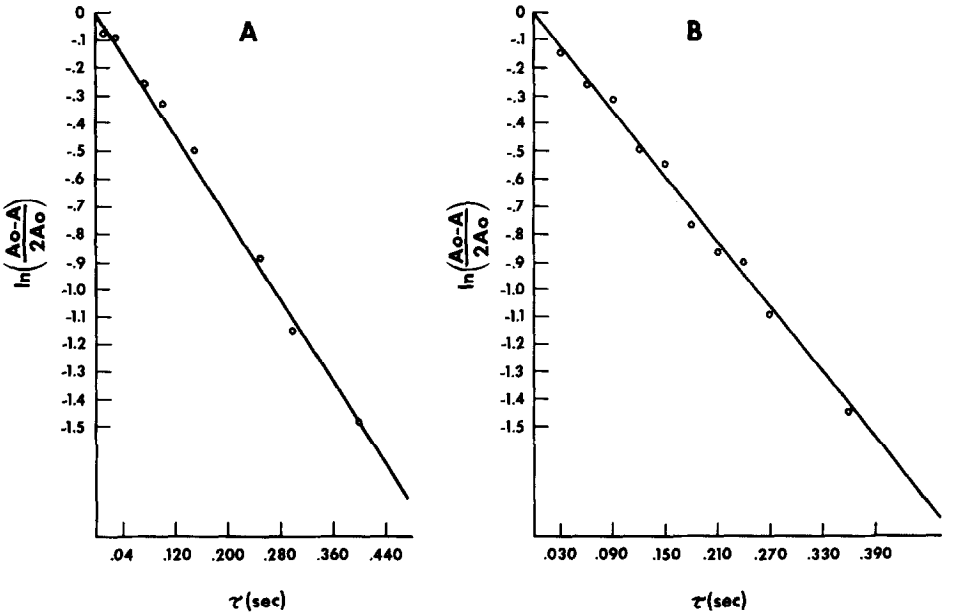

Fig. 3. Plots of amplitude versus τ . τ is the waiting period between the 180° and 90° pulse. A) Sonicated vesicles in which each point was transformed after 50 scans using an exponential filter. B) Unsonicated vesicles in which each point was transformed after 100 scans using an exponential filter.

shown in Figure 3. In both cases the correlation coefficient of the least squares was better than 0.995.

In anisotropic systems such as trimethyl ammonium micelles and polystyrene polymers, T_1 values reflecting ^{13}C spin-lattice relaxation of protonated carbons are dominated by segmental motion (18,19). Under these conditions, T_1 is inversely proportional to an effective correlation time which is dominated by the rotational correlation time due to segmental motion (18). It thus seems reasonable to conclude that the similarity of T_1 values in the two phospholipid bilayer systems reflects an equivalence of segmental motions of the N-methyl residues. Chan has shown in bilayer systems, that T_2^* measurements are sensitive to slower molecular motions (20). This is especially true of those motions perpendicular to the main axis of orientation of the molecule in the bilayer. In addition, he shows that segmental motions have little effect on the T_2^* times. If differences in molecular packing exist between single-walled vesicles and large multi-lamellar liposomes, then these differences would be reflected in anisotropic motions allowed the molecule in the two bilayer systems. A more ordered packing arrangement would have greater restrictions on the movement of the molecule. The rotational correlation of the perpendicular motions would increase, thereby giving rise to a shorter T_2^* time. The fact that the T_2^* for the

large multi-lamellar liposomes is smaller than the corresponding value for the small single-walled vesicles is consistent with evidence that suggests that the small structures are the more disordered (4,21).

It is, however, possible that the differences in T_2^* values might arise from chemical shift non-equivalence. Thus the broader line of the multi-lamellar liposome spectrum could be an envelope of slight 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 multi-lamellar liposomes the majority of the phospholipid is in concentric lamellae of essentially infinite radii of curvature. On the basis of purely geometrical considerations, Chan has shown that differences in curvature have a negligible effect on packing when the bilayer radius is larger than about 800Å (4). In contrast to this situation, the effect of bilayer curvature is maximal in small, single-walled vesicles (22), and thus chemical shift non-equivalence should be greatest in these structures. Although proton NMR studies have demonstrated a chemical shift non-equivalence between phosphatidylcholine N-methyl protons on the inner and outer bilayer faces in single-walled vesicles of minimum radius (4,23), no such shift is seen in the ^{13}C NMR resonance. Since no chemical shift non-equivalence 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 non-equivalence can make a substantial contribution to line width under the unfavorable conditions existing in multi-lamellar liposomes. It thus seems reasonable that the differences in N-methyl ^{13}C T_2^* values obtained in the two bilayer systems does, in fact, reflect differences in molecular organization.

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