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¹⁹F-NMR OF FLUORINE LABELED ACYL CHAINS IN PHOSPHOLIPID BILAYER VESICLES

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¹⁹F-labeled phospholipids, L- α -bis-(ω -fluoro palmitoyl)phosphatidylcholine, L- α -bis-(12,12-difluoro stearoyl)phosphatidylcholine and L- α -bis-(6,6-difluoro palmitoyl)phosphatidylcholine, were incorporated in phospholipid vesicles by sonication of aqueous lipid emulsions. Vesicles were prepared both from the pure fluorine substituted phospholipids as well as from lipid mixtures obtained by combining the fluorine substituted lipids with the synthetic phospholipids, L- α -dilauroyl-, L- α -dimyristoyl-, L- α -dipalmitoyl- and L- α -distearoyl-phosphatidylcholine. Characterization by gel permeation chromatography showed that stable unilamellar vesicles with diameters of \sim 200 Å could be obtained with a minimum or absence of multilamellar material. The vesicles give rise to two ¹⁹F resonances in most cases as observed previously [K.J. Longmuir and F.W. Dahlquist, Proc. Natl. Acad. Sci. U.S.A., 73 (1976) 2716]. The chemical shift differences undergo systematic changes that confirm the interpretations that the dual ¹⁹F resonances arise from the inner and outer halves of the vesicle bilayer. The shift separation increases systematically as the fluorine label is positioned closer to the phospholipid headgroup and decreases systematically with increasing temperature. Both observations agree with what is currently known about phospholipid vesicle structure. Anomalous results are obtained with DSPC as host vesicle since only a single resonance of inbedded fluorinated phospholipids is found.

Keywords: ¹⁹F-NMR; phospholipid; vesicle; bilayer.

Introduction

¹⁹F-NMR of synthetically labeled phospholipids has been found to be particularly useful in the study of lipid protein interactions [2,3]. The small size of the fluorine atom makes it especially suitable as a relatively non-perturbing replacement probe for the H atom [4] although some recent studies [5] suggest that it can alter the

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Abbreviations: DAST, diethylaminosulfur trifluoride; DLPC, L- α -dilauroyl-phosphatidylcholine; DMPC, L- α -dimyristoyl-PC; DPPC, L- α -dipalmitoyl-PC; DSPC, L- α -distearoyl-PC; 6,6-F-DPPC, L- α -bis-(6,6-difluoro palmitoyl)-PC; 12,12-F-DSPC, L- α -bis-(12,12-difluoro stearoyl)-PC; ω F-DPPC, L- α -bis-(ω -fluoropalmitoyl)-PC; PC, phosphatidylcholine.

thermotropic phase behavior of synthetic model membranes. The fluorine probe has been used successfully in a number of studies both of phospholipids [3,6,7] as well as fluorinated fatty acids [8] incorporated into lipid bilayer vesicles. Moreover, the motions of the fluorine labeled acyl chains and the relationship to the ^{19}F spin-spin and spin-lattice relaxation times have been established for multilamellar [6,7] as well as vesicle systems [6].

Initial studies of the ^{19}F -NMR of a synthetically labeled phospholipid in a lipid vesicle revealed an unusual splitting of the fluorine resonance [1]. The two signals were interpreted as arising from fluorine substituted phospholipid molecules on either half of the vesicle bilayer. The asymmetry created by differences in packing between the inner and outer halves of the vesicle as a result of the bilayer curvature, has been suggested as the rationale for the chemical shift differences observed.

In preliminary experiments with 12,12-F-DSPC, incorporated into a DMPC vesicle, we have also observed the two resonances [9]. We sought, therefore, to examine this asymmetry induced effect in a variety of systems as a function of the position of the ^{19}F label, the nature of the host phospholipid, and the temperature.

Experimental

Materials

Preparation of the keto esters (6-ketomethylpalmitate). Adipic acid was converted into its half acid ester in 60% yield by the procedure of Swann et al. [10]. The corresponding acid chloride was then prepared with phosphorus pentachloride in 75% yield as described by Cason [11]. Reaction of this half ester-acid chloride (ω -carbomethoxy pentanoyl chloride) with didecylcadmium prepared from decylbromide Grignard reagent and cadmium chloride in ethyl ether gave a 42% yield of 6-ketomethylpalmitate [11] (m.p. 34–35°C, recrystallized from hexane).

(12-Ketomethylstearate). 12-Hydroxystearic acid was converted to 12-ketostearic acid (m.p. = 82–83°C, 75% ethanol-water) via the CrO_3 /pyridine oxidation [12] in 62% yield. This compound was evaluated for positional purity by converting it to the corresponding keto methylester. The mass spectral analysis of this derivative showed that the original 12-hydroxystearic acid was >92% isomerically pure. Major ions were observed at 242 and 128 characteristic of fragments corresponding to $\text{C}_{14}\text{H}_{26}\text{O}_3^+$ and $\text{C}_8\text{H}_{16}\text{O}^+$, respectively.

Diethylaminosulfur trifluoride (DAST) was prepared by the procedure of Middleton and Bingham [13].

Preparation of the gem-difluoro fatty acids-general procedure. Approximately 6 mmol of keto ester was added to a Teflon* screw top bottle (previously dried and flushed with nitrogen) containing 5 ml (38 mmol) of DAST and 5 ml of Freon 11

which was previously cooled to 0°C. The reaction mixture was slowly warmed to room temperature, stirred, and allowed to react for 9 days. The reaction was quenched by slowly adding the reaction mixture to 250 ml of an ice-water slurry. Caution! This reaction is highly exothermic and can be dangerous if addition is too rapid. Hydrolysis of the final product was effected by the addition of 3 ml of 50% NaOH and 100 ml of methanol and heating at 65°C for 5 h. The reaction mixture was then acidified with 20% HCl, extracted three times with 60 ml portions of methylene chloride, and dried over Na₂SO₄. Evaporation of the solvent on a rotary film evaporator gave a 90–95% crude yield of gem difluoro fatty acid. Subsequent recrystallization of these products from acetone gave materials with a purity >95% by glc. The melting points of the derived fatty acids are: 6,6-difluoropalmitic, 76–77°C and 12,12-difluorostearic, 70.5–72.0°C.

Preparation of 16-fluoropalmitic acid (ω -F palmitic acid) methyl juniperate (16-hydroxymethylpalmitate). To a solution of 30 ml of anhydrous methanol containing 5% sulfuric acid w/w was added 8 g of 16-hexadecanolide (Farchan Division; Storey Chemical Corp.). The reaction mixture was refluxed for 2 h, solvent removed, and the residue recrystallized from methanol, m.p. = 93–94°C.

(16-Fluoropalmitic acid). 16-Hydroxymethylpalmitate was fluorinated with DAST except that its fluorination was complete within 2 h. Workup was the same as described above for the keto acid derivatives. The melting point of the final product was 73.5–74°C (90% hexane-acetone).

Preparation of 1,2-bis(difluoroalkyl) phosphatidylcholines. All purified fluorinated fatty acids were coupled to L- α -glycerophosphorylcholine-CdCl₂ complex via the acid anhydride procedure of Khorana et al. [14]. The purity of each phospholipid was verified on silica gel tlc eluted with CHCl₃/MeOH/H₂O (65:25:4). In all cases, only trace amounts of lysophospholipid were observed in the final purified material.

The non-fluorine labeled phospholipids (DPLC, DMPC, DPPC and DSPC) were obtained from Sigma Chemical Company as the L- α form, synthetic, approx. 99% pure and were used without further purification. All other chemicals were the highest purity obtainable from commercial sources.

Preparation of the phospholipid vesicles. The vesicles were prepared as follows: (1) the host lipid material (~50 mg) containing 2–15 mg of the fluorinated phospholipid was added to a round bottom flask and dissolved in a small amount of CHCl₃. (For those preparations with no host, just the fluorinated phospholipid was used.) A thin film was cast on the interior surface of the flask by evaporating the chloroform in a stream of dry N₂ gas while rapidly rotating the flask. The flask was then evacuated for 2 h to remove residual traces of CHCl₃. (2) The lipid film was then emulsified with 1 ml D₂O containing 0.05 mM Tris (pH 7.5) (no correction for deuterium was made), and 0.1 M KCl. (3) The emulsion was then sonicated using a Branson Model 180 sonicator with microprobe tip at a temperature well above the thermal transition temperature of the host lipid (30°C for DLPC and DMPC, 50°C

for DPPC and 70°C for DSPC). Sonication was carried out until the emulsion mixture was translucent; roughly 30–60 min. (4) The NMR spectra were obtained on the samples at temperatures above the thermal transition temperatures, care being taken not to let the sample temperatures fall below these points.

Chromatography. The vesicle preparations were characterized using gel permeation chromatography with Sepharose 4B (cross-linked) as the support material. With Sepharose 2B (cross-linked), the vesicle fraction eluted very close to the total volume and with Sepharose 6B (cross-linked), very close to the void volume. The column used was approx. 30 cm high with an inside diameter of 1.5 cm. Total volume of the packed column was approx. 18 ml with a void volume of approx. 7 ml as determined by indigo blue and blue dextran, respectively. The column was loaded with 0.5 ml of the sonicated emulsion and the sample eluted with H₂O buffer of the same pH, buffering capacity, and ionic strength as the sample D₂O buffer. The effluent was monitored with a refractive index detector in a flow through cell.

NMR measurements. All NMR measurements were carried out on a Bruker WH-90 spectrometer operating at a frequency of 84.7 MHz at variable temperatures on approx. 0.5 ml of sample in D₂O buffer in a 5 mm sample tube. The D₂O provided the lock signal. A narrow capillary containing 1% by volume of trifluoroacetic acid in H₂O was inserted into the sample tube and was used as a reference signal. Accumulations (5000–10 000) of 90° pulses with a repetition rate of 1–2 s were sufficient for each measurement depending on the line width of the fluorinated phospholipid to produce spectra with good signal-to-noise ratios. Sweep widths were generally 5000–10 000 Hz using 8 K data points of memory. Broad band proton decoupling with maximum power (~2 W) was used throughout.

Results and discussion

Chromatography

Typical chromatographs obtained with several preparations are shown in Fig. 1. The negative peaks at the total volume represent the D₂O in the injected samples. In both cases, a single broad peak was obtained with a $K_{av} \sim 0.45$ which indicates roughly a mean diameter of about 200 Å [15]. Occasionally, a small peak was observed at the void volume as an indication of a very small percentage of multilamellar material. Most vesicle preparations were chromatographed in order to insure that they did not contain any significant amounts of the large particles.

All sonicated preparations regardless of host vesicle, fluorinated phospholipid, or the ratio of the two, fall within a very narrow range with regard to their K_{av} values. This result indicates that a fairly uniformly sized vesicle was obtained for all preparations. Since the chromatography was carried out at room temperature, those host phospholipids whose thermal transition temperatures were above room

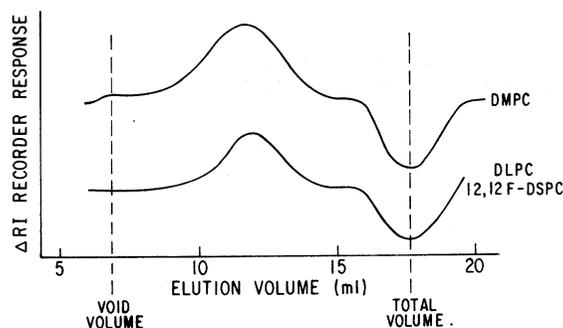


Fig. 1. Sepharose 4B gel permeation chromatographs of vesicle preparations. Void volume and total volume are approx. 7 and 18 ml, respectively. The upper trace is for pure DMPC vesicles; the lower trace is for 12,12-F-DSPC in a DLPC host vesicle.

temperature tended to aggregate in the column. The results in these cases were not as clear cut as with the lower 'melting' phospholipids. What was certain, however, was that if the sonications were carried out above the transition temperature, nearly optically clear preparations could be obtained and remained that way if the temperature was not permitted to fall below the transition temperature.

NMR

The three fluorinated phospholipids provide a range of phospholipids in which the fluorine label was positioned down the chain from the six position to the terminal end. Consequently, the motional environment of the fluorine can be examined as a function of distance along the chain. The four host phospholipids chosen, DLPC, DMPC, DPPC and DSPC provide a range of differing chain lengths as well as differing thermal transition temperatures. The composite data for both the line widths of the ^{19}F NMR resonances as well as the chemical shift separations between the two fluorine resonances are given in Table I.

The rather remarkable observations by Longmuir and Dahlquist [1] of the split ^{19}F resonances of the 7,7- and 12,12-difluorinated phospholipids in vesicle systems have been extended in the present study. All three fluorine substituted phospholipids exhibited the double resonance both in the pure vesicle state as well as in the host vesicles with the following exceptions. With DSPC as host, none of the substituted phospholipids showed the dual fluorine resonance. $\omega\text{F-DPPC}$ in the pure vesicle also showed only a single peak. In general, the separation of the ^{19}F resonances was independent of the ratio of host lipid to the fluorine labeled phospholipid. The ratio was varied from 2:1 up to 20:1 host to label in several instances with no significant differences in the results.

TABLE I

¹⁹F-CHEMICAL SHIFT DIFFERENCES, $\Delta\delta$ BETWEEN SEPARATE FLUORINE SUBSTITUTED PHOSPHOLIPID RESONANCES AND CORRESPONDING LINE WIDTHS, $W_{1/2}$

Values (Hz) are reproducible to within $\pm 7\%$. Full width at half height. Values are reproducible to within $\pm 10\%$.

Host	Temp. (°C)	¹⁹ F substituted phospholipids					
		ω_F		12,12		6,6	
		$\Delta\delta$	$W_{1/2}$	$\Delta\delta$	$W_{1/2}$	$\Delta\delta$	$W_{1/2}$
DLPC	30	16	4.7	44	26	90	28
	50	14	2.8	33	14	49	19
	70	15	3.2	24	16	29	15
DMPC	30	21	5.4	86	32	122	56
	50	15	3.2	59	17	52	19
	70	17	3.2	32	12	24	14
No Host	50	0 ^a		32	22	25	23
	70	0		15	10	13	14
DPPC	50	23	4.1	56	18	50	32
	70	20	3.4	31	11	14	17
DSPC	70	0	4.0	0		0	26
	85	0	4.4	0		0	22

^aA value of zero indicates that only a single resonance is observed. In several cases, poor signal-to-noise precluded a measurement of the line widths.

The separate ¹⁹F resonance signals were relatively narrow as seen by others for ¹⁹F substituted phospholipids in vesicles [3,6]. For example, compare the corresponding spin-spin relaxation rate ($R_2 = 100/s$) for L- α -bis (8,8-difluoro palmitoyl) phosphatidylcholine [3,6] at 50°C to the value, $R_2 = 60/s$ for 6,6-F-DPPC in the present study. As seen in Table I, the line widths generally increase as the substituted fluorine atom is incorporated closer to the phospholipid head groups. For example, in the DMPC host vesicle at 30°C, the values are 5.4, 32 and 56 Hz for the fluorine substituted at the ω -, 12- and 6-positions, respectively. These values reflect the greater local motions of the fluorine atom as it is positioned down the phospholipid chain toward the end of the molecule. This variation of line width with position along the chain parallels that observed for ¹³C T_1 relaxation times as a function of position down the chain in NMR studies of phospholipid vesicles [16]. The resonances for the ω position are extremely narrow and approach those seen for fluorine

substituted phospholipids in CHCl_3 solution [6]. Also, as expected, the line widths decrease generally with increasing temperature for any one combination. For example, 6,6-F-DPPC in the DMPC host vesicle gives rise to line widths of 56, 19 and 14 Hz at 30°C, 50°C and 70°C, respectively. These results also indicate the greater mobility with increasing temperature.

The chemical shift differences between the two separated resonances observed show several systematic variations which give further evidence that the two signals originate from the asymmetry of the outside versus the inside portion of the vesicle. The first of these variations is the increase in chemical shift difference as the fluorine label is positioned closer to the phospholipid head group. The largest separations are shown for the 6,6-F-DPPC (up to 122 Hz in the DMPC host at 30°C), ranging down through the 12,12-F-DPPC, to the ω F-DPPC which shows the smallest separations. Even though this relationship does not hold strictly in every case, the overall trend is very clear.

These data are consistent with the previous interpretation that the two resonances observed arise from fluorine substituted molecules on opposite sides of the bilayers. The effects of the asymmetry of inner and outer would be greatest nearest the head group and should diminish as the fluorine moves down the chain. At the ω position, the environment of the fluorine atoms should be nearly identical and show the least asymmetry, and consequently, the smallest chemical shift difference as observed.

In fact, it is rather remarkable that the ω -substituted fluorines in the phospholipid chain show a chemical shift separation for different sides of the bilayer. One would suspect that the ω -fluorine atom on either side would move in approximately the same molecular space and have on the average the same chemical shift. That a difference occurs indicates that their average environments are not identical. However, since no doubling is observed for the fluorine resonance of the pure ω F-DPPC vesicle, the environment seen there by the fluorine atoms on either side of the bilayer must be nearly identical.

The other somewhat surprising result is that only a single ^{19}F resonance is found for the fluorinated phospholipids when the host vesicle is DSPC. Line widths, although slightly larger in all cases, are comparable to those for the phospholipids in the other host vesicles. At the same time, the temperature of the preparations was kept clearly above the transition temperatures of DSPC of roughly 55°C [18]. Nevertheless, DSPC has the highest transition temperature of all the host phospholipids. Nearly identical environments in both halves of the bilayer as shown by the lack of splitting in the ^{19}F resonance indicates that phase separation may be occurring in these vesicles. If the ^{19}F substituted phospholipids were separating into patches, the uniformity of the environments on both bilayer halves could easily mask the bilayer asymmetry. Phase separation among dissimilar phospholipids occurs in many instances, particularly in multilamellar system, but generally this happens below the gel-to-liquid crystal transition temperature [18]. Furthermore,

fluorinated phospholipids have been shown by differential scanning calorimetry to form highly nonideal mixtures with their parent compounds, another indication of possible phase separation [19].

Interestingly, when the stearyl chains are fluorine substituted, namely in our experiments with isomerically pure 12,12-F-DSPC, the dual ^{19}F resonance was found. Temperature effects appear to be intimately bound up with these phenomena because the presence of fluorine in the phospholipid significantly lowers the gel-to-liquid crystal transition temperature [19].

The other systematic variation noted is a monotonic decrease in the chemical shift separation with increasing temperature. A number of the appropriate data points have been selected from Table I and are presented in Fig. 2.

There is a sharp decline in the separation for 6,6-F-DPPC with increasing temperature, less so for 12,12-F-DPPC and no variation for ω F-DPPC. The variation with temperature correlates very well with the variation in size of the vesicle with temperature. From a study on the variations of vesicle architecture with temperatures, it was found [17] that vesicle size increased significantly with increase in temperature. In Table II are given some of the appropriate values, showing an increase in both the inner and outer radii. While a substantial part of the increase in both inner and outer radii occurs over the gel-to-liquid crystal transition at approx. 24°C [18], the vesicle continues to expand as the temperature increases beyond

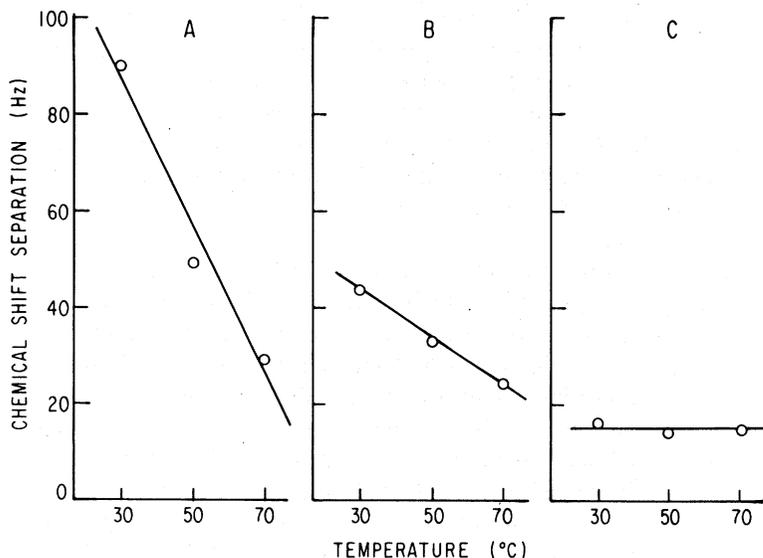


Fig. 2. Chemical shift separations of the two ^{19}F -NMR resonances of labeled phospholipids as a function of temperature. (A) 6,6-F-DPPC; (B) 12,12-F-DSPC; (C) ω F-DPPC, all in DLPC host vesicles.

TABLE II
 DIMENSIONS OF DIMYRISTOYL PHOSPHATIDYLCHOLINE BILAYER VESICLES
 Dimensions expressed as Å [15].

	Temp.			
	15°C	20°C	25°C	30°C
Outer radius	95.2	93.2	108.7	112.2
Inner radius	44.5	54.5	77.2	82.4
Ratio (outer to inner)	2.14	1.71	1.41	1.36

that point. Moreover, as the vesicle size increases, the ratio of outer to inner radius decreases, resulting in a decrease in the asymmetry of outer to inner half of the bilayer. The decrease in the chemical shift difference with temperature reflects the lessening of the differential asymmetry. Also, the decrease is the greatest for the fluorine closest to the polar head groups, whereas ω -substituted phospholipids are the least affected by change in vesicle size as anticipated.

Conclusion

Fluorine substituted phospholipids contained in bilayer vesicles exhibit an extraordinary dual resonance attributed to fluorine atoms on different halves of the bilayer. The separate resonances are interpreted as arising from the natural asymmetry of the inner and outer layers of the vesicle. This has been amply demonstrated by the present study especially by the systematic variations in the chemical shift differences of the two fluorine signals: an increase in the difference as the fluorine is positioned closer to the polar head group and a decrease in the separation as the temperature is increased. Both observations are in accord with the interpretation of the chemical shift difference as outlined above, the latter, because of the increase in vesicle size with increasing temperature. Unusual results are obtained when DSPC is the host vesicle since no doubling of the ^{19}F resonance is observed. While there are indications that phase separation is occurring in these cases, the results cannot as yet be interpreted unambiguously. Nevertheless, fluorine substituted phospholipids are potentially powerful probes of protein lipid interactions within model membrane bilayer vesicles, since in principle, one can assess the effects of the interaction within specific halves of the bilayer.

Acknowledgments

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