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A ^{13}C-NMR STUDY OF 10,12-TRICOSADIYNOIC ACID AND THE CORRESPONDING PHOSPHOLIPID AND PHOSPHOLIPID POLYMER

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Diacetylene phospholipids are presently being studied because of their potential to polymerise in vesicles, multilayers and natural biomembranes. ^{13}C-NMR spectra and spin-lattice relaxation times have now been obtained of a diacetylene phospholipid present in a sonicated dispersion in water. Similar data have been obtained of a monoacetylene phospholipid and a saturated phospholipid. For further comparison the spectrum of a diacetylenic fatty acid in benzene-d_6 was also examined and relaxation data obtained. A comparison of the various relaxation data provides an indication of the restricted motion associated with the two conjugated triple bonds of the diacetylene phospholipid within the lipid bilayer structure. A proximity interaction between diacetylene groups occurs and a conformation for the diacetylene part of the lipid in the bilayer is deduced. The ^{13}C-NMR spectrum of a soluble phospholipid polymer in C^2HCl_3, obtained by ultraviolet irradiation of the diacetylene phospholipid, shows that the two conjugated triple bonds of the monomer is replaced in the polymer by an alternating double and triple bonded conjugated structure.

Introduction

Studies by several research groups have led to the development for investigating protein-lipid interactions of phospholipid analogues that can be cross-linked within biomembrane structures [1,2]. Phospholipid molecules which can undergo extensive cross-linking and polymerisation have also been recently introduced [3,4]. The phospholipids are synthesized so as to contain diacetylenic groups in one or both of the acyl chains. In this case, the polymerisation process is initiated by ultraviolet light or X-ray irradiation.

These diacetylenic phospholipids can be dispersed in water to form vesicles and can be used to reconstitute intrinsic membrane proteins so as to restore normal enzyme activity. In some cases feeding the acetylenic fatty acid to auxotrophic microorganisms leads to the biosynthesis of glycolipids and phospholipids containing the appropriate diacetylene group. Polymerisation of the biomembranes of the cell can than be induced [3]. It is interesting to note that diacetylenic lipids also occur naturally in certain plant systems.

NMR spectroscopy has been widely applied to the study of phospholipids in solution, as vesicles, and present in biomembrane structure to gain information about the structure, conformation, interactions and dynamic behaviour [5–7] and various nuclei have been investigated including ^{1}H, ^{13}C, ^{2}H and ^{31}P nuclei.

The object of the present report is to describe

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our spectroscopic studies using ^{13}C-NMR spectroscopy of a diacetylenic fatty acid and phospholipid before and after polymerisation and to compare the corresponding spectra with those of saturated and unsaturated fatty acids and also other phospholipids. In order to interpret the spectroscopic data lanthanide-induced shift (LiS), relaxation time measurements and spectra at different magnetic fields have been obtained.

Materials and Methods

The synthesis of diacetylenic phospholipids and fatty acids has been described elsewhere [3]. The acetylenic phospholipids have been synthesized from the corresponding fatty acids using anhydrides [8] or imidazolides [9] for the esterification of the cadmium chloride complex of glycerophosphocholine from natural sources [10]. Stearolic acid (octadec-8-ynoic acid) was prepared from oleic acid by bromination and dehydrobromination. Stearic acid and dipalmitoylglycerophosphocholine (DPPC) were purchased from Sigma. Yb(fod)$_3$ (fod = 1,1,1,2,2,2,3-heptafluoro-7,7-dimethyloctane-4,6-dionate) from NMR Ltd. was stored over P_2O_5 before using it. Phospholipid dispersions were prepared by agitating the desired amount of freeze-dried pure phospholipid above its transition temperature with water or a buffer and subsequently sonicating it under nitrogen, in a probe sonicator. The resultant translucent dispersion was centrifuged in order to remove any titanium particles. Dispersions for T_1 measurements were made in 40 mM phosphate buffer, pH = 7, containing 50% ^2H_2O and a small amount of EDTA Na_2, and degassed with a stream of nitrogen. Dispersions of diacetylenic phospholipid were kept away from light and the experiments were run at 60°C. After the experiments were completed no changes in the purity of the sample could be detected by TLC. To obtain the T_1 values, an inversion-recovery sequence $(\pi\text{-}\tau\text{-}(\pi/2)\text{-}T)_x$ was used, and the resulting intensities for different delays τ between the π and $\pi/2$ pulses were fitted directly using a non-linear least square fitting programme to the following equation;

$$y = Ae^{-t/T_1} + B$$

The use of three independent parameters (A, B, T_1) has been shown to minimise the effect of pulse imperfections and other systematic errors [11].

Spectra of polymer, dissolved in C^2HCl_3 and prepared by ultraviolet irradiation of diacetylene phospholipid in water and purified by passing it through Sephadex LH-60, were obtained at 50 MHz using a spherical microcell and block averaging to overcome dynamic range problems.

Spectra were run on the following instruments: Brucker HFX-90 (22.63 MHz), WP-250 (62.89 MHz) and WP-400 (100 MHz) belonging to the University of London, Intercollegiate Research Service and also the Brucker WM-200 (50.31 MHz) wide-bore, WP-360 (90.52 MHz) and Varian XL-200 (50.31 MHz) instruments.

Results

The ^{13}C-NMR spectrum of tricosa-10,12-diynoic acid were recorded at 62.89 MHz in benzene-d_6 solution. At this magnetic field the perturbation due to the diacetylene group allows the resolution of 21 resonances for the 23 carbon atoms of the molecule. The assignment was made by comparison with saturated fatty acids, using lanthanide-induced shift (LiS) data and relaxation time measurements to assign the otherwise very similar methylene resonances. Chemical shifts, respect to TMS, are reported in Table I along with the measured T_1 and the lanthanide shift extrapolated to 0.5 equivalents of Yb(fod)$_3$.

Lanthanide-induced shifts were measured for a series of saturated and unsaturated fatty acids. The relative shifts of the methylene resonances are very similar in all the cases with significant differences only for the unsaturated carbons.

The ^{13}C-NMR spectra at 62.89 MHz of 1,2-di(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine in benzene-d_6 solution and in sonicated aqueous dispersions are presented in Fig. 1. The assignment of resonances has been made with the aid of the off-resonance spectra and by comparison with chemical shifts reported in the literature for saturated phospholipids [12] and diacetylenic compounds [13]. The assignment of the methylene resonances which become resolved due to the effect of the diacetylene group in the middle of the fatty acyl chain was made using the corresponding

TABLE I

CHEMICAL SHIFTS, RELAXATION TIMES AND LANTHANIDE-INDUCED SHIFT FOR TRICOSA-10,12-DIYNOIC ACID IN BENZENE SOLUTION

(δ = 0 with respect to TMS)

Carbon No.	δ(ppm)	$\Delta\delta$(0.5 eq)	T_1(s)
1(COOH)	181.50	–	2.09
2	34.81	–	0.77
3	25.45	–	0.99
4	29.75	13.85	1.00
5	29.87	9.45	1.20
6	29.68	5.65	1.17 [a]
7	29.53	3.50	1.16
8	29.18	2.35	1.08
9	19.95	1.40	0.95
10 C	78.14	0.87	10.15
11 C	67.34	0.70	17.59
12 C	67.29	0.48	18.68
13 C	78.26	0.38	10.46
14	19.95	0.25	0.95
15	29.27	0.25	1.37
16	29.68	–	– [a]
17	30.02	–	1.95
18	30.43	–	2.39
19	30.55	–	2.55
20	30.31	–	2.99
21	32.87	–	3.79
22	23.69	–	4.67
23	14.94	–	6.30

[a] Overlapping resonance

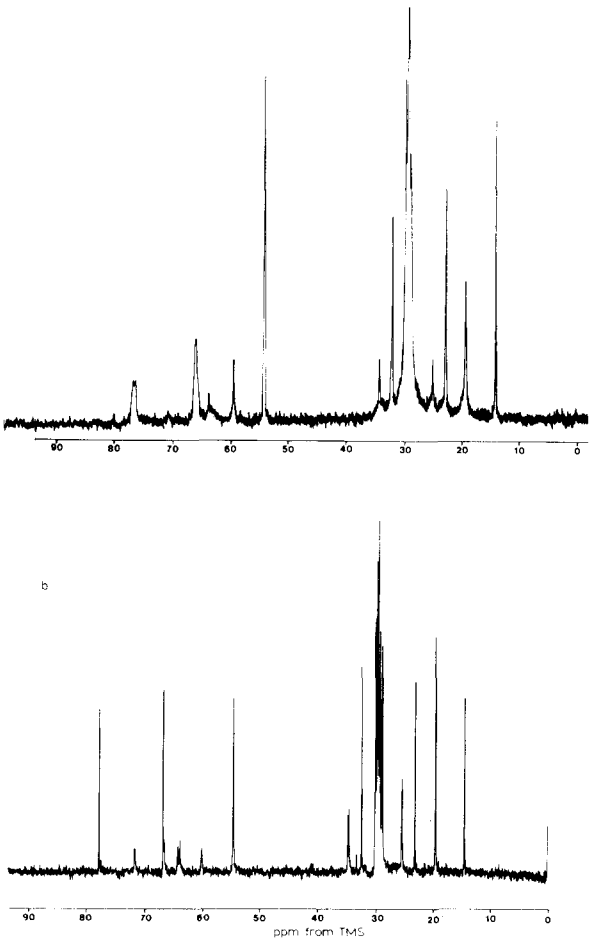

Fig. 1. ^{13}C-NMR (62.89 MHz) spectra of a diacetylenic phospholipid (a) in sonicated water dispersion and (b) in benzene solution 30° pulses, 1.08 s interpulse time. The peaks corresponding to the carbonyl group are not shown.

fatty acid spectrum previously assigned. The result is shown in Table II.

Apart from the changes in relative intensities of the different peaks, which can be explained by the different relaxation times in the two systems, obtained from the benzene solution and the aqueous dispersion, the main difference between the spectra consists of the shielding and linewidth of the acetylenic resonances. No precise linewidth measurements could be made for the internal acetylenic carbon (at approx. 67 ppm) because of overlapping with headgroup resonances but the linewidth for the external acetylenic carbon (at approx. 78 ppm) could be determined at several magnetic fields. The result is shown in Table III.

Spin-lattice relaxation times were measured at 50 MHz for sonicated dispersions of the diacetylenic phospholipid (1,2-di(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine), a monoacetylenic phospholipid (1,2-dioctadecynoyl-*sn*-glycero-3-phosphocholine) and a saturated phospholipid (DPPC) under identical conditions. The results are presented in Table IV.

Diacetylenic phospholipid vesicles were polymerised below their transition temperature and the structure of the isolated polymer was confirmed by ^{13}C-NMR. Chemical shifts with respect to TMS are reported in Table V.

TABLE II

ASSIGNMENTS OF THE RESONANCES RESOLVED IN THE SPECTRUM OF DIACETYLENIC PHOSPHOLIPIDS IN BENZENE SOLUTION

Peak No.	Chemical shift [a] δ(ppm)	Assignment
1	173.49	$-{}^{1}\underline{C}OO-$ [b]
2	173.35	$-{}^{10}\underline{C}\equiv C-C\equiv{}^{13}\underline{C}-$
3	77.65	
4	71.59	$-\underline{C}HOCOR$
5	66.75	$-C\equiv{}^{11}\underline{C}-{}^{12}\underline{C}\equiv C-$
6	66.55	$-\underline{C}H_2N^+$
7	64.16	$-\underline{C}H_2OP-$glycerol
8	63.80	$-\underline{C}H_2OCOR$
9	59.96	$-\underline{C}H_2OP$ choline
10	54.47	$-N^+(\underline{C}H_3)_3$
11	34.68	$-{}^{2}\underline{C}H_2-COO-$ [b]
12	34.49	
13	32.35	$-{}^{21}\underline{C}H_2-CH_2-CH_3$
14	30.04	$-{}^{19}\underline{C}H_2$
15	29.94	$-{}^{18}\underline{C}H_2$
16	29.79 (2C)	$-{}^{20}\underline{C}H_2+?$ [c]
17	29.69	? [c]
18	29.52 (2C)	? [c]
19	29.35	$-{}^{16}\underline{C}H_2-CH_2-CH_2-C\equiv C-C\equiv C-\ldots$ COO
20	29.23	$-C\equiv C-C\equiv C-CH_2-CH_2-{}^{7}\underline{C}H_2\ldots\ldots$ COO
21	28.97	$-{}^{15}\underline{C}H_2-CH_2-C\equiv C-C\equiv C\ldots\ldots\ldots$ COO
22	28.83	$-C\equiv C-C\equiv C-CH_2-{}^{8}\underline{C}H_2\ldots\ldots\ldots$ COO
23	25.48	$-{}^{3}\underline{C}H_2-CH_2-COO-$ [b]
24	25.38	
25	23.16	$-{}^{22}\underline{C}H_2-CH_3$
26	19.55	$-{}^{9}\underline{C}H_2-C\equiv C-C\equiv C-{}^{14}\underline{C}H_2$
27	19.49	
28	14.41	$-{}^{23}\underline{C}H_3$

[a] Relative to TMS. The values reported correspond to a solution in benzene.

[b] The two resonances arise from the two acyl chain of the phospholipid molecules.

[c] Methylene resonances were assigned by comparison with free acid. Carbons 4, 5, 6 and 17 could not be assigned in this way.

TABLE III

LINEWIDTH OF THE ACETYLENIC RESONANCE AT ~ 78 ppm AT 1,2-DI(TRICOSA-10,12-DIYNOYL)-*sn*-GLYCERO-3-PHOSPHOCHOLINE AT DIFFERENT SPECTROMETER FREQUENCIES

Spectrometer frequency	$\Delta\nu_{1/2}$ (Hz)	$\Delta\nu_{1/2}$ (ppm)
22.63	27	1.2
50.31	60	1.2
62.89	75	1.2
90.52	105	1.2

Discussion

The resolution of most of the methylene resonances in the ^{13}C-NMR spectrum of the diacetylenic acid is due to the shielding effect of the triple bonds. This effect is very important for methylenes directly bound to the triple bond and amounts to -10.8 ppm. The diamagnetic anisotropy of the triple bonds could explain the shielding observed for carbons up to four bonds away from the triple bond. However, a quantitative agreement with distances and angles derived from

TABLE IV

RELAXATION TIMES FOR AQUEOUS DISPERSIONS OF PHOSPHOLIPIDS

Temp = 333 K, T_1 in seconds.

Assignment	T_1 (s), acyl group		
	Hexadecanoyl	Octadec-8-ynoyl	Tricosa-10,12-diynoyl
Triple bonds	0	1	2
$\underline{C}OO-$	3.00	3.50	3.00 ± 0.50
$\underline{C}H_2-COO-$	0.33	0.30	0.37 ± 0.06
$\underline{C}H_2-CH_2-COO-$	0.40	0.40	0.37 ± 0.10
$(CH_2)_n$	0.60	0.80	0.50
$\underline{C}H_2-C\equiv C$	–	0.60	0.28 ± 0.02
$C\equiv C$	–	3.40	$1.3/1.2 \pm 0.10$
$\underline{C}H_2-CH_2-CH_3$	1.18	0.98	1.19 ± 0.07
$\underline{C}H_2-CH_3$	1.80	2.00	1.90 ± 0.10
$\underline{C}H_3$	5.00	5.40	3.70 ± 0.30
$(CH_3)_3N^+$	0.83	1.20	1.09 ± 0.03
CH_2-O-P-choline	0.50	0.50	0.40 ± 0.10

molecular models using the equation of McConnell and Robertson [14] was not obtained for the rest of carbons affected; rather, an exponentially decreasing effect with distance was observed. The relaxation times for the diacetylenic fatty acid show a gradient from the carboxyl group to the methyl end. This is typical of amphiphilic molecules in solution. In this case a second gradient can be seen extending from the carbon next to the diacetylene group towards the carboxyl end. This gradient, along with the very short relaxation times of the methylenes next to the triple bonds, strongly suggests that reorientation of the rigid diacetylenic group is difficult even in benzene solution.

TABLE V

CHEMICAL SHIFTS WITH RESPECT TO TMS OF A POLYDIACETYLENIC PHOSPHOLIPID IN $CdCl_3$

Assignment	δ (ppm)	Assignment	δ (ppm)
$\underline{C}OO$	173.6	$(CH_2)_n$	28.2
$-\underline{C}=\underline{C}-C\equiv C-$	134.0	$\underline{C}H_2-CH_2-COO$	24.0
$-C=C-\underline{C}\equiv\underline{C}-$	108.6	$\underline{C}H_2-CH_3$	21.7
$(\underline{C}H_3)_3N^+$	53.6	$\underline{C}H_2-C=C$	18.4
$\underline{C}H_2-COO$	36.2	$\underline{C}H_3$	13.2
$\underline{C}H_2-CH_2-CH_3$	31.1		

A comparison between relaxation time measurements and shift reagents as assignment tools for long, flexible molecules can be made in this case. Clearly, for molecules with a single binding site, the use of lanthanides is the method of choice up to 14–15 carbon atoms distant from this site. This is particularly the case if superposition of resonances takes place. It should be pointed out that with the diacetylene fatty acid that the existence of a second relaxation time gradient, extending from the centre of the chain, could lead to a wrong assignment for carbons 5 to 8. Conversely, for longer distances the differences in lanthanide-induced shift become comparable with the experimental error. However, significant relaxation time differences occur and allow an unambiguous assignment for the rest of the carbon atoms in the chain.

Spectra of sonicated dispersions have been shown to have relatively narrow lines due to averaging of dipolar interaction by fast tumbling of the phospholipid vesicles and rapid lateral diffusion of the highly curved bilayer. The difference in linewidth with respect to the solution spectra can be attributed to a T_2 effect indicating a reduction of the motion of the sonicated vesicles. Indeed, some authors have used linewidth measurements to study this motion [15,16]. The appearance of the unusual

broadening in the acetylenic resonance could be attributed to a very slow motion association with the rigidity of the two conjugated triple bonds. An alternative explanation could come from the existence of proximity effects between diacetylene groups of the oriented acyl chains in the bilayer structure of the phospholipid dispersions. The existence of proximity interactions in acetylenic compounds has been proposed by Charrier et al. [17] in cyclic diacetylenic compounds in which a shift of the acetylenic resonances could be observed as the size of the ring was decreased. In their case, the effect of ring strain could not be accounted for, leaving some doubt about their interpretation. We believe that our observations in a strain-free system confirm their suggestions.

In our case, the distinction between a relaxation effect and a proximity interaction was made by recording the spectra at four different magnetic fields. As shown in Table III, the linewidths follow a linear relationship with the spectrometer frequency. That is a clear indication that the observed broadening effect arises from the superimposition of lines having different chemical shifts. The spread of the lines, giving only a broad envelope, indicates a range of relative orientations and distances, each of them giving a different interaction between diacetylene groups and interconvening at a rate that is slow on the NMR time scale. The field dependence experiments allow us to discard a third broadening mechanism involving a chemical shift anisotropy (CSA) relaxation pathway. If the broadening arose from a decreased T_2 due to the existence of a dominant mechanism of chemical shift anisotropy one would expect a quadratic dependence on the magnetic field which is not the case in the experiments shown in Table III.

The ^{13}C-NMR spectrum, at 50 MHz, of 1-acyl-2 (tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine (i.e. the phospholipid containing a diacetylenic group in only acyl chain) presents in linewidth for the external acetylenic resonance which is half of the corresponding value reported in Table III, but still is the broadest peak in the spectrum. This indicates that both intra and intermolecular interactions take place in diacetylenic phospholipid vesicles.

The observed interaction is a shielding effect. The unperturbed acetylenic resonances of the phospholipid dissolved in benzene lies to the low-field side of the broad band absorbed with the phospholipid in aqueous dispersion. From the known diamagnetic anisotropy of triple bonds, a head to head or head to tail arrangement of the diacetylene groups must be occurring. From the unequivalence of the 1- and 2-acyl chain of phospholipids and molecular model building the following structure for the arrangement of diacetylenic phospholipids in bilayers can be suggested (Fig. 2).

It should be noted that this represents a chiral arrangement of the diacetylenes produced by the stiffness of the diacetylenes and the packing of the aliphatic chains.

Some insight into the effects of triple bonds on the dynamic properties of phospholipids can be obtained from T_1 measurements presented in Table IV. The following features are apparent:

(i) Relaxation times for the headgroup and re-

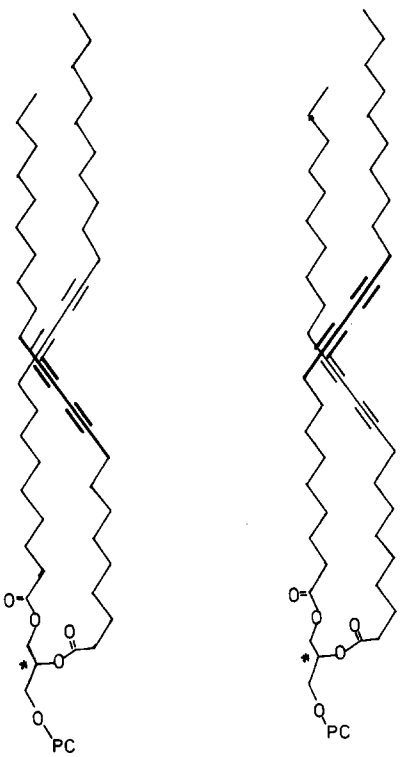

Fig. 2. Schematic diagram of two possible diastereomeric conformations of the aliphatic chains of 1,2-di(tricosa-10,12-diynoyl)-*sn*-3-glycero-3-phosphocholine in phospholipid vesicles.

solved resonances in the acyl chain show very similar values for the three phospholipids studied. A mobility gradient is apparent from the long T_1 values for the resonances near the terminal methyl group and the short relaxation times from the methylenes adjacent to the headgroup.

(ii) Quaternary carbons present comparatively long relaxation times, reflecting the dependence of dipole-dipole relaxation with the inverse of the cube of the distance to the nearest protons. Nevertheless, it should be noted that whilst the relaxation times for the acetylenic and carboxylic carbons are similar in the monoacetylenic phospholipid, in the case of the diacetylenic phospholipid the acetylenic carbons relax much faster.

(iii) Relaxation times for the methylenes adjacent to the triple bonds in the diacetylenic phospholipid are among the shortest in the molecule. The value found for the corresponding methylenes in the monoacetylenic phospholipid is similar to the value for the metylene groups which are in the centre of the acyl chains.

The fast relaxation of the six carbons in the segment comprising the diacetylene group and the adjacent methylenes indicate a very small degree of mobility of this portion of the chain within the lipid bilayer. The monoacetylenic phospholipid presents much less restriction of mobility of this part of the chain within the lipid bilayer structure. The existence of proximity interactions which are not averaged out also indicates that a restriction of mobility in the diacetylene region of the chains is occurring.

The picture that emerges from the ^{13}C-NMR study presented in this paper shows that although the six carbon long, rigid diacetylene group presents difficulties for reorientation inside the bilayer, the dynamic properties of the rest of the phospholipid are not much altered.

The ^{13}C-NMR spectrum of the phospholipid polymer reported in Table V shows resonances corresponding to an olefinic and a highly deshielded acetylenic carbon. This assignment was made by comparison with a reported ^{13}C-NMR spectrum of a soluble, urethane substituted, diacetylenic polymer [19]. These resonances are in contrast to the two diacetylenic resonances which occur with the unpolymerised phospholipid as shown in Table II.

From this we deduce that the structure of the diacetylenic phospholipid polymer consists of a conjugated backbone, formed by alternating double and triple bonds. This conclusion is consistent with recent Raman spectroscopic studies [18].

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