Biochimica et Biophysica Acta, 419 (1976) 1–18

BBA 77163

PROTON MAGNETIC RELAXATION STUDIES OF MIXED PHOSPHATIDYLCHOLINE/FATTY ACID AND MIXED PHOSPHATIDYLCHOLINE BIMOLECULAR BILAYERS

F. PODO* and J. K. BLASIE

Department of Biophysics and Physical Biochemistry, Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. (U.S.A.)

(Received February 3rd, 1975)
(Revised manuscript received June 12th, 1975)

SUMMARY

High resolution proton spin-lattice relaxation times (T_1), spin-spin relaxation times (T_2) and resonance linewidths were measured above the gel-to-liquid crystal transition temperature (T_m), in phosphatidylcholine bilayers possessing various degrees of intramolecular motional anisotropy at the level of various alkyl chain proton groups. The experiments were designed to test the hypothesis that coupled *trans-gauche* isomerizations along the chains can be responsible for the anisotropic motion of phosphatidylcholine proton groups in bilayer membranes (Horwitz, A. F., Horsley, W. J. and Klein, M. P. (1972) Proc. Natl. Acad. Sci. U.S. 69, 590). Systematic series of structural perturbations of the bilayer were achieved in mixed phosphatidylcholine/fatty acid and in mixed phosphatidylcholine bilayers where the degree of motional anisotropy of the chains' proton groups was gradually reduced by progressively increasing the chain length disparity of the two components. The systematic T_1 and T_2 variations observed in these systems were interpreted on the basis of the Woessner's treatment for computing the relaxation times of a spin pair reorienting randomly about an axis which, in turn, tumbles randomly (Woessner, D. E. (1962) J. Chem. Phys. 36, 1). The results confirmed in a qualitative sense the original hypothesis made by Horwitz et al. The time-averaged structural interpretations suggested by the magnetic relaxation studies are in agreement with low-angle X-ray diffraction results obtained below T_m.

In addition, the T_1 values evaluated at various temperatures in dipalmitoyl phosphatidylcholine vesicles incorporated with either ^{2}H-labeled or unlabeled palmitic acid chains indicated that the average intermolecular contribution to the spin-lattice relaxation rate of the proton groups of the phosphatidylcholine chains appears comparable to the intramolecular term at temperatures moderately higher than T_m, but becomes less and less important as the temperature is further increased above the thermal transition.

---

* Present address: Istituto Superiore di Sanità, Physics Laboratory, Rome, Italy.

INTRODUCTION

Nuclear magnetic resonance techniques are nowadays extensively used for assessing the mobility and molecular arrangement of the molecular components in natural and model membranes. The potentiality and limitations of these studies have been recently discussed [1, 2]. Temperature- and frequency-dependence measurements, carried out on phosphatidylcholine vesicles [2], have shown that motional anisotropy plays an important role in determining both spin-lattice relaxation times (T_1) and linewidths ($\Delta\nu_{\frac{1}{2}}$) of the various proton groups' resonances. One-correlation-time models [3–5] are therefore inadequate for a correct interpretation of the magnetic relaxation behaviour in these systems [1, 6]. A more sophisticated approach is offered by the Woessner's treatment [7] of the magnetic relaxation of a proton pair undergoing a rotational Brownian motion about an axis, which in turn may undergo random reorientations at a different rate. Specifically, partial rotations of proton pairs about the chemical bonds and reorientations of the rotational axes (as would occur through *trans-gauche* and *gauche-gauche* isomerizations [6, 8]) seem able to provide a first-approximation of the bilayer's dynamic structure, as detected through nuclear magnetic relaxation properties. This approach has recently appeared useful for assessing the dynamic perturbations induced on dipalmitoyl phosphatidylcholine bilayers by the incorporation of additional molecular components (e.g. chlorophyll [9] or fluorescent probes [10]).

These considerations have suggested a further testing of this anisotropic motional model in mixed lipid bilayers, where the degree of motional anisotropy was gradually modified through the addition of lipid components of progressively shorter chain length into the host bilayer. ESR experiments, carried out on planar bilayers of egg phosphatidylcholine containing both lauric acid and a steroid spin-label probe, have shown, for instance, that a larger distribution of orientations was made available to the probe's molecular axis as the content of lauric acid was increased [11]. On the other hand, the hydration of the polar groups is not significantly affected by the mixing of phosphatidylcholine molecules having different chain length [12], which suggests that the main structural modifications occurring in the mixed bilayer are essentially localised within the membrane hydrocarbon core.

Direct T_2 measurements on the various proton resonances appeared to be essential for assessing the relative importance of the magnetic dipole-dipole interactions versus other factors possibly contributing to the resonance linewidths, such as unresolved chemical shift spread and resonance multiplicity.

Finally, ^{2}H-labeling of one of the lipid bilayer components appeared to be useful for assessing the importance of the intermolecular contributions to the proton spin-lattice relaxation at various temperatures.

This paper describes proton resonance linewidths, spin-spin (T_2) and spin-lattice (T_1) relaxation studies carried out at various temperatures above the thermal transition of the chains on phosphatidylcholine/fatty acid mixed bilayers (dipalmitoyl phosphatidylcholine vesicles containing, respectively, stearic acid, unlabeled vs-[^{2}H] palmitic acid, and myristic acid) and on phosphatidylcholine/phosphatidylcholine mixed bilayers (distearoyl/dipalmitoyl and distearoyl/dimyristoyl phosphatidylcholines). The motion of the alkyl chains' proton groups in the phospholipid bilayers incorporated with moderately shorter-chain lipids becomes more isotropic, a dynamic

modification interpreted in terms of an increased rate of the isomeric fluctuations of the methylene groups around the C-C bonds, leading to an average increase in the number of *gauche* isomers along the alkyl chains. This interpretation is also supported by low-angle X-ray diffraction results, obtained on leaflets of the same systems below the chains' thermal transition. These studies show that, under incorporation of moderately shorter-chain components, the bilayer thickness decreases in a manner which requires a reduction in the time-averaged extension of the longer alkyl chain species.

Part of this work has been briefly reported elsewhere [13].

MATERIALS AND METHODS

Synthetic β,γ-dipalmitoyl L-α-phosphatidylcholine purchased from Calbiochem was used for preparing the mixed phosphatidylcholine/fatty acid samples; stearic, palmitic and myristic acids were purchased from Applied Science Laboratories, Inc.

[^{2}H]Palmitic acid was supplied by Merck and Company, Inc. [^{2}H]Chloroform (99.8 % minimum isotopic purity) was purchased from Thompson-Packard, Inc.

Synthetic β-γ-distearoyl, β-γ-dipalmitoyl and β-γ-dimyristoyl L-α-phosphatidylcholines used for the experiments on mixed phosphatidylcholine bilayers were purchased from Applied Science Laboratories, Inc.

The appropriate amounts of the lipid components were dissolved in [^{2}H]-chloroform and successively dried, under vacuum, on the walls of a test tube.

Mixed bilayers of dipalmitoyl phosphatidylcholine and fatty acid at mol ratio 2 : 1 and 1 : 1 (38.1 mM phosphatidylcholine) were prepared by suspending the dry components in a ^2H_2O buffer (45 mM NaCl, 30 mM sodium acetate, 5 mM sodium phosphate, p^{2}H (20 °C) = 7.6).

Bilayers of distearoyl phosphatidylcholine and mixed bilayers of distearoyl/dipalmitoyl phosphatidylcholine and distearoyl/dimyristoyl phosphatidylcholine (1 : 1) (total phosphatidylcholine 30 mM) were prepared by suspending the dry components in a similar ^2H_2O buffer (45 mM NaCl, 30 mM sodium acetate, 5 mM sodium/potassium phosphates, p^{2}H (20 °C) = 7.9).

Small-diameter single-bilayer lipid vesicles were obtained by a 2-min low-power sonication at 20 kHz, applied with a sonifier cell disruptor (Branson model W185), under N_2 flow, to the coarse, previously degassed dispersions. During the sonication the temperature of the sample was maintained above the fatty-acid chain thermal transition. The formation of considerable thermal gradients between the sample and the bath was avoided by alternating 15-s sonication with 15-s waiting intervals.

The sonicated samples of mixed phosphatidylcholine bilayers were kept above 60 °C during the time required for the NMR experiments. The fact that the samples were never allowed to undergo the "fluid" to "fluid and solid" thermal transition avoided possible intravesicular phase separation phenomena [12, 14] in order to maintain the ideal mixing of the two lipid components as created within the vesicles by the applied sonication.

NMR spectra and pulsed NMR measurements of the relaxation times of the individual protons [15] were obtained at 220 MHz using a Varian HR-220 spectrom-

eter, equipped with a frequency sweep unit, operating in both the Continuous Wave [16] and Fourier Transform [17] mode. The probe temperature was controlled by means of a Varian Model V-4340 variable temperature accessory. The width and timing of the transmitter radio-frequency pulses were controlled with a Varian 620-I computer.

For the T_1 measurements a radio-frequency pulse sequence ($\pi/2$, homospoil, τ, $\pi/2$, free induction decay, homospoil) was employed [18] and the pulse sequence was repeated as many times as necessary for a given delay time, τ, in order to achieve the desired signal-to-noise ratio (typically approx. 10 transients).

High-resolution T_2 measurements were carried out on non-spinning samples, employing Hahn's pulse sequence [19] ($\pi/2$ -τ-π-τ-free induction decay), repeated as many times as necessary for achieving the desired signal-to-noise ratio; a delay time of 30 s between successive sequences allowed the spin system to completely recover both its transverse and its longitudinal equilibrium magnetization. Owing to instrumental limitations, the τ values could not be set at values shorter than 10 ms. The phase modulation of the spectra was avoided by using the absolute value representation of the Fourier transformed spectra [20]. The application of this technique was justified by the fact that the various signals in the spin-echo spectra either were well separated or did not overlap to any significant extent.

The same computer Varian 620-I was used for collecting the free induction decay signals from the receiver and for applying the Fourier transform operation to transform the free induction decays into the frequency domain spectra [21, 22]. The high resolution T_1 and T_2 values were calculated from the partially relaxed spectra, with the use of a PDP-10 computer, by analysing the line amplitudes S as a function of τ and 2τ, respectively. Typically 20–25 points were taken for each experiment. For T_2 measurements the amplitudes S (2τ) were analysed in terms of either single or multiple spin-spin relaxation rates; in the cases where more than one rate was required for fitting the data, the respective spin fractions contributing to each relaxation component were estimated. When the spin-echo amplitude of the signals studied as functions of τ exhibited a modulation [23], superimposed on the damping due to the spin-spin relaxation, the damping component was easily separated and analysed by computer in terms of either single or multiple spin-spin relaxation rates. This modulation (modulation frequency approx. 120 Hz) occurred only in the cases of the chains' terminal -CH_3 group and the choline's $N^+(CH_3)_3$ group spin-spin relaxation and not in the case of the chains' -(CH_2)-$_{4-(n-1)}$ groups' spin-spin relaxation.

The errors in the T_1 values determined for the chains' terminal methyl groups are estimated to range between 15 and 20 %, because of the partial overlapping of this signal with the resonance of the main chain methylene groups. The other T_1 values and the T_2 determinations are estimated to have errors of ± 5 % and ± 10 %, respectively.

RESULTS

Above the thermal transition of the chains, dipalmitoyl phosphatidylcholine vesicles incorporated with fatty acids exhibit proton magnetic resonance spectra analogous to those of pure phosphatidylcholine vesicles [2], with the only exception that due to selective line-broadenings, some of the minor proton resonances disappear

under the noise in the dipalmitoyl phosphatidylcholine/fatty acid systems. The main resonances dominating the spectra are those due to the choline $N^+(CH_3)_3$ and the chains' $-(CH_2)-_{4-(n-1)}$ and terminal $-CH_3$ groups; clearly detectable resonances are also observed for the choline NCH_2 and the first methylene group in the fatty acid chain, CH_2-COO. The chemical shifts of the dipalmitoyl phosphatidylcholine vesicles incorporated with any of the three fatty acids coincide with those reported for pure dipalmitoyl phosphatidylcholine vesicles in ref. 2. Owing to the low intensity of the NCH_2 and CH_2-COO signals, the determination of their T_1 and T_2 values appeared to be difficult. It should also be noted that, within the limits of experimental detectability, the methylene band profile for $-(CH_2)-_{4-(n-1)}$ was maintained essentially unaltered in the T_1 and T_2 partially relaxed spectra in both the dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine/fatty acid systems (as well as in the mixed phosphatidylcholine bilayers discussed later in this section).

The spin-lattice relaxation rates determined for the dipalmitoyl phosphatidylcholine bilayers containing, respectively, myristic, palmitic and stearic acids are plotted in Fig. 1 as functions of the reciprocal temperature, and compared with those of pure dipalmitoyl phosphatidylcholine vesicles. The introduction of stearic acid into dipalmitoyl phosphatidylcholine bilayers does not modify to any considerable extent

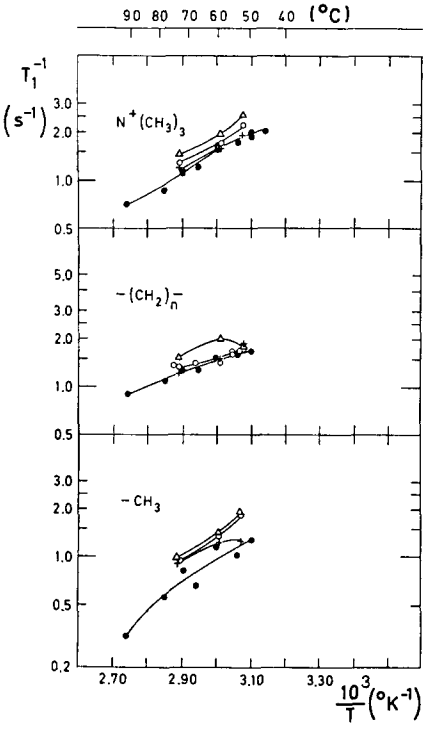

Fig. 1. Proton spin-lattice relaxation rates at 220 MHz of the choline trimethylammonium group, $N^+(CH_3)_3$, the chains' methylene groups, $-(CH_2)-_{4-(n-1)}$, and the chains' terminal methyl groups, $-CH_3$, for dipalmitoyl phosphatidylcholine vesicles (●) and containing stearic (+), palmitic (○) and myristic (△) acids (mol ratio 2 : 1). Measurements were carried out above the chains' thermal transition. Experimental errors are mentioned in the text.

TABLE I

220 MHz PMR LINEWIDTHS AND SPIN-LATTICE RELAXATION RATES OF DIPALMITOYL PHOSPHATIDYLCHOLINE VESICLES CONTAINING STEARIC ACID AND MYRISTIC ACID

1, dipalmitoyl phosphatidylcholine/stearic acid (2 : 1); 2, dipalmitoyl phosphatidylcholine/myristic acid (2 : 1). Accuracy of resonance linewidth measurements is ± 1 Hz for $N^+(CH_3)_3$ and ± 2 Hz for $-(CH_2)_{4\ (n-1)}$ and CH_3.

System	Temperature (°C)	$\Delta\nu_{\frac{1}{2}}$ (Hz)			$T_1^{-1}(s^{-1})$		
		$-N^+(CH_3)_3$	$-(CH_2)_{4\ (n-1)}$	$-CH_3$	$N^+(CH_3)_3$	$-(CH_2)_{4\ (n-1)}$	$-CH_3$
1	59	7	45	20	1.54	1.47	1.27
	73	5	35	20	1.20	1.22	0.94
2	59	7	35	20	1.90	1.96	1.42
	73	5	25	20	1.47	1.52	1.01

the spin-lattice relaxation rates of either the $N^+(CH_3)_3$ or the chains' methylene groups; upon further reducing the chain length of the introduced fatty acid (palmitic and myristic acid) the relaxation rates of these groups appear progressively increased, the strongest deviations from the behaviour of the pure phosphatidylcholine vesicles being those observed in the system containing myristic acid. In particular the slight rate increment observed with increasing temperature for the chains' methylene groups of the dipalmitoyl phosphatidylcholine/palmitic acid system has been confirmed in two separate sets of experiments, carried out with different samples. Within the rather large experimental errors, the modifications induced by the free fatty acid on the relaxation rates of the chains' terminal methyl groups follow substantially the same trend already observed for the other two groups, stearic acid still appearing the least perturbing; the effects of palmitic acid and myristic acid on this group appear, however, to be practically identical.

The resonance linewidths observed in the systems dipalmitoyl phosphatidylcholine/stearic acid and dipalmitoyl phosphatidylcholine/myristic acid at 59 and 73 °C (Table I) show that a selective line-narrowing occurs at the level of the chains' methylene resonance band, on going from the bilayers containing stearic to those containing myristic acid.

High resolution spin-lattice relaxation times, measured above the chains' thermal transition for dipalmitoyl phosphatidylcholine vesicles incorporated, respec-

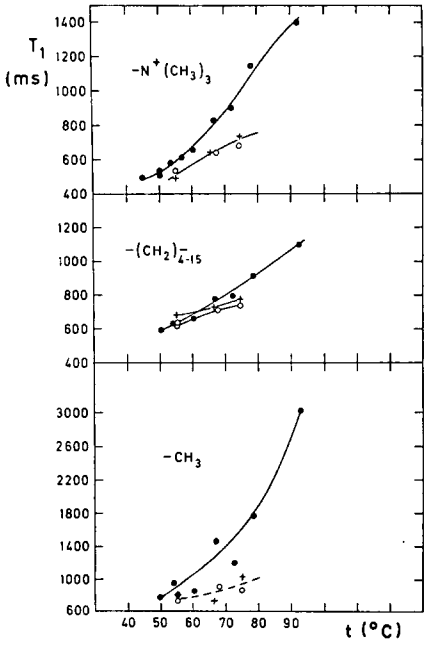

Fig. 2. Proton spin-lattice relaxation rates at 220 MHz of the choline trimethylammonium group, $N^+(CH_3)_3$, the chains' methylene groups, $-(CH_2)_{4-15}$ and the terminal methyl groups, $-CH_3$, measured above the chains' thermal transition on dipalmitoyl phosphatidylcholine vesicles (●) and vesicles containing unlabeled (○) and ^{2}H-labeled (+) free palmitic acid chains (mol ratio phosphatidylcholine/fatty acid, 2 : 1).

TABLE II

220 MHz PMR SPIN-LATTICE RELAXATION RATES OF THE CHAINS' $-(CH_2)-_{4-15}$ GROUPS IN DIPALMITOYL PHOSPHATIDYLCHOLINE (DPPC) VESICLES CONTAINING UNLABELED FREE (PA) AND [^{2}H]PALMITIC ACID ([^{2}H]PA) CHAINS

Temperature (°C)	T_1^{-1} (s^{-1})		Temperature (°C)	T_1^{-1} (s^{-1})	
	DPPC : PA (2 : 1)	DPPC : [^{2}H]PA (2 : 1)		DPPC : PA (1 : 1)	DPPC : [^{2}H]PA (1 : 1)
55	1.60	1.47	55	1.90	1.36
66	1.43	1.39	60	1.46	1.43
74.5	1.34	1.31	71.5	1.28	1.18

tively, with ^2H-labeled and unlabeled palmitic acid chains (mol ratio 2 : 1) are plotted vs temperature in Fig. 2. Within experimental errors, the relaxation times of both the choline $N^+(CH_3)_3$ and the chains' terminal methyl groups are practically the same in the systems incorporated with either unlabeled or [^2H]palmitic acid chains. Systematic, although slight, T_1 increments are observed instead, upon ^2H-labeling of palmitic acid, for the chains' methylene groups at the explored temperatures. Measurements carried out also on systems containing palmitic acid at the mol ratio 1 : 1 (Table II) further substantiate this conclusion.

High-resolution proton magnetic resonance spectra exhibited above the chains' thermal transition by aqueous suspensions of distearoyl phosphatidylcholine vesicles (Fig. 3) are closely analogous to those obtained under identical temperature conditions by dipalmitoyl phosphatidylcholine vesicles [2], with the exception that

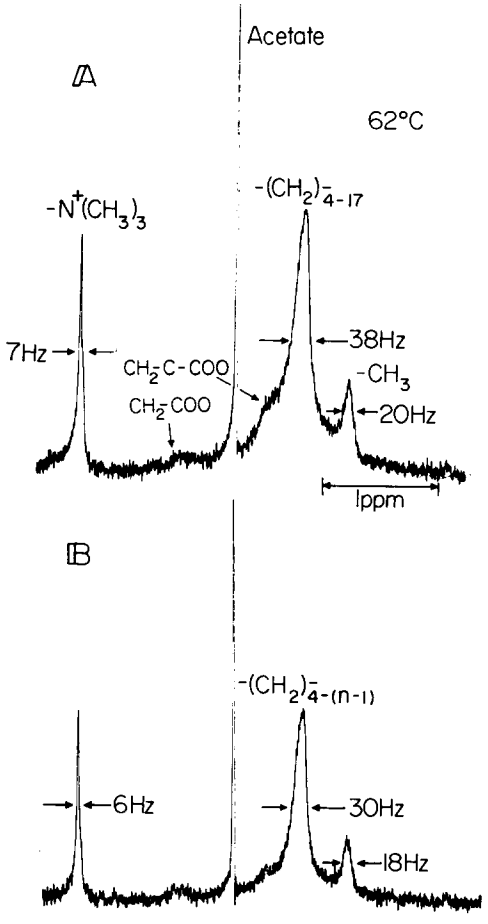

Fig. 3. 220 MHz proton magnetic resonance spectra of aqueous dispersions of (A) distearoyl phosphatidylcholine and (B) distearoyl phosphatidylcholine/dimiristoyl phosphatidylcholine (mol ratio 1 : 1). The spectra have been obtained above the thermal transition of the chains with the continuous wave technique, under slow passage conditions, at a radiofrequency level well below the saturation of any of the phosphatidylcholine signals. The linewidths of the main resonances are indicated.

TABLE III

220 MHz PMR RESONANCE LINEWIDTHS, SPIN-LATTICE AND SPIN-SPIN RELAXATION TIMES, MEASURED ON VESICLES OF DISTEAROYL PHOSPHATIDYLCHOLINE, DISTEAROYL/DIPALMITOYL PHOSPHATIDYLCHOLINES (1 : 1) AND DISTEAROYL/DIMYRISTOYL PHOSPHATIDYLCHOLINES (1 : 1)

In the cases where the two-rate analysis gave a better fit of the data, the results of both the one- and the two-rate analysis are reported. The proton fractions with the longer and the shorter T_2 values are indicated, respectively, in parenthesis.

Sample phosphatidylcholine	Temp. (°C)	$N^+(CH_3)_3$				$-(CH_2)_{4-(n-1)}$				$-CH_3$			
		T_1 (ms)	T_2 (ms) 1-rate	T_2 (ms) 2-rate	$\Delta\nu_{\frac{1}{2}}$ (Hz)	T_1	T_2 1-rate	T_2 2-rate	$\Delta\nu_{\frac{1}{2}}$ (Hz)	T_1	T_2 1-rate	T_2 2-rate	$\Delta\nu_{\frac{1}{2}}$ (Hz)
Distearoyl	62	700	174	195 (87 %) 63 (13 %)	7 ± 1	695		46 (~20 %) 5 (~80 %)*	38 ± 2	1090	29	–	19 ± 2
Distearoyl/dipalmitoyl	62	670	167	–	6 ± 1	700	37	94 (19 %) 22 (81 %)	35 ± 2	1110	34	–	15 ± 2
Distearoyl/dimyristoyl	62	725	212	334 (62 %) 70 (38 %)	6 ± 1	715	42	412 (8 %) 30 (92 %)	30 ± 2	1110	32	–	18 ± 2
Distearoyl	74	900	232	–	7 ± 1	800	27	63 (14 %) 10.4 (86 %)	30 ± 2	1590	22	59 (15 %) 11 (85 %)	17 ± 2
Distearoyl/dipalmitoyl	74	952	274	–	5 ± 1	820	52	452 (7 %) 41 (93 %)	25 ± 2	1490	29	123 (7 %) 7.6 (93 %)	16 ± 2
Distearoyl/dimyristoyl	74	962	254	312 (23 %) 8.2 (77 %)	4 ± 1	855	52	52 (100 %)	25 ± 2	1265	20	37 (13 %) 8.1 (87 %)	15 ± 2

* Estimated by the reduction of the "echo" signal intensity after 2τ = 20 ms in the Hahn sequence (see text).

the resonance linewidths of the alkyl chains appear broader in the former system (cf. Table III of the present work and Table III of ref. 2).

The resonance linewidths (Table III) appear systematically narrowed by incorporation of shorter-chain phosphatidylcholines into the distearoyl phosphatidylcholine bilayer, the most significant effects being those shown by the chains' $-(CH_2)-_{4-(n-1)}$ resonance band. In particular at 62 °C dimyristoyl phosphatidylcholine appears to be more effective than dipalmitoyl phosphatidylcholine in producing a selective narrowing of this resonance band.

Direct T_2 measurements (Table III) show that a monotonic correlation exists at various temperatures between the predominant spin-spin relaxation times and resonance linewidths of the chains' methylene groups, longer T_2 values corresponding to narrower linewidths. (This correlation has also been verified in the dipalmitoyl phosphatidylcholine systems containing fatty acids.)

As shown in Table III, the spin-lattice relaxation times of the choline $N^+(CH_3)_3$ and the chains' $-(CH_2)-_{4-(n-1)}$ groups appear either generally unaltered or only slightly increased on going from pure distearoyl phosphatidylcholine bilayers to distearoyl phosphatidylcholine bilayers containing either dipalmitoyl or dimyristoyl phosphatidylcholine, the highest observed increment being of the order of 5 %. The spin-lattice relaxation behaviour of the chains' terminal methyl group is not significantly different in the three systems at 62 °C, while at 74 °C the T_1 rate appears progressively increased (within 20 %) as the chain length of the incorporated phosphatidylcholine becomes shorter.

DISCUSSION

A. Intrachain magnetic dipole-dipole interactions

Proton magnetic resonance linewidths in phosphatidylcholine vesicles may depend in principle on both the vesicle size and the correlation times of anisotropic intramolecular motions [24, 25, 2]. Selective linewidth changes (namely exhibited by only one or a few chemical groups along the phosphatidylcholine molecule) cannot be simply related to a modified vesicle size; they might therefore be interpreted in terms of a modified degree of motional anisotropy of that proton group, provided that the linewidths are mainly determined by the spin-spin relaxation rate, instead of by chemical shift spread of unresolved resonances [6]. In both the mixed phosphatidylcholine and the phosphatidylcholine/fatty acid bilayers this condition is generally fulfilled. The following monotonic correlations have in fact been observed at the various temperatures, between the spin-spin relaxation rates and the resonance linewidths.

(a) The linewidths of the $N^+(CH_3)_3$ signals correspond closely to those predicted for two partially overlapping lines, located 3–4 Hz apart (as those arising from the headgroups on the "inner" and the "outer" surfaces of the bilayer vesicle, respectively [26]), and having a linewidth of $1/\pi T_2$ Hz each.

(b) The linewidths of the terminal methyl groups are practically those expected for an alkane methyl triplet (J-coupled to the vicinal methylene unit) whose components have linewidths of $1/\pi T_2$ Hz each.

(c) Within the limits of experimental detectability the various components of the $-(CH_2)-_{4-(n-1)}$ band exhibit practically the same T_1 (and T_2) values; the band

profile is in fact maintained substantially unaltered in the T_1 (and T_2) partially relaxed spectra even when using π, τ, $\pi/2$ pulse sequences for the T_1 measurements.

(d) A monotonic correlation exists at the various temperatures between the predominant spin-spin relaxation time and the resonance linewidth of the chains' $-(CH_2)-_{4-(n-1)}$ band. In particular, in the systems where (as in the dipalmitoyl phosphatidylcholine/fatty acid and pure distearoyl phosphatidylcholine bilayers) a one-rate analysis represents a reasonable fit of the experimental spin-echo decay for the chain methylene resonance band, the observed $1/\pi T_2$ values represent 20–50 % of the observed linewidths. It should also be explicitly noted that after a value of 2τ = 20 ms (the minimum "refocusing" time that could be set in our spectrometer for the Hahn sequence) the chains' methylene resonance is already reduced to less than 20 % of its original intensity: more than 80 % of the protons are estimated therefore to have a relaxation time $T_2 < 5$ ms. This result is in agreement with previous measurements by Horwitz et al. [8]. Under the conditions where the shorter T_2 value is increased (in the systems distearoyl/dipalmitoyl and distearoyl/dimyristoyl phosphatidylcholine) a quantitative estimate of the proton fractions having, respectively, the longer and the shorter spin-spin relaxation times, is easily allowed by a computer two-rate analysis (no cases were recorded where a three-rate analysis gave a meaningfully better fit of the data).

These facts suggest the conclusion that the linewidth of the chains' $-(CH_2)-_{4-(n-1)}$ resonance band in phosphatidylcholine vesicles is mainly determined by the predominant spin-spin relaxation rate, instead of by chemical shift spread of the various $-(CH_2)-$ groups along the chains. Analogous conclusion is valid for the $N-CH_3$ and terminal CH_3 signals, provided that the non-equivalence of the inner and outer groups is considered for the former and the triplet nature of the proton signal for the latter.

On the other hand, not only linewidths but also T_1 values are generally affected by the inherent motional anisotropy of the system [2]. It has been shown that at least a two-correlation-time treatment is required for (a) interpreting the temperature dependence trends of the T_1 values observed at 60 and 220 MHz, for the various proton groups of dipalmitoyl phosphatidylcholine vesicles [2] and (b) for reconciling the following phenomena, generally occurring in phosphatidylcholine bilayers: the general increase of the T_1 values with increasing temperature [27, 28, 2] and the high T_1/T_2 ratios [8, 28]. A reasonable, although simplified, approach for interpreting these data is offered by the previously mentioned Woessner model [7]. Intramolecular rotation around the bonds (average correlation time τ'_c) is expected to affect primarily the spin-lattice relaxation, while the slower segmental reorientation of the bond axes (average correlation time τ''_c) affects mainly the spin-spin relaxation [29]. However, depending upon the value of the ratio τ'_c/τ''_c (typically ranging from 10^3 to about 10), segmental reorientations may also strongly affect the T_1 values, as indicated by Eqns 1, 2 and 21 of ref. 7 and by the schematic drawings of Fig. 5 of ref. 2. In particular, the following two cases will be considered here.

(a) A dynamical perturbation of the bilayer producing an increased T_1^{-1} paralleled by an increased T_2 (decreased linewidth) can be interpreted as a reduction of the τ'_c/τ''_c ratio, namely in terms of a reduced degree of motional anisotropy (as illustrated in the schematic curves of Fig. 4, drawn under the condition that the average rotational correlation time is not significantly perturbed).

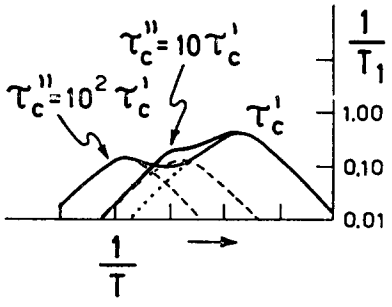

Fig. 4. Schematic theoretical curves representing the expected temperature dependence of the spin-lattice relaxation rates for a proton pair undergoing anisotropic motion. The latter is here characterized by two correlation times, τ'_c and τ''_c. The dotted lines represent the separate contributions to the spin-lattice relaxation due to the fast and the slow motions, respectively. For simplicity's sake, equal activation energies are assumed for the contributions from the two motions, while the internuclear vector has been taken as perpendicular to the rotation axis [7]. The example is intended to show that a ten-fold decrease of τ''_c causes a considerable increase in the resulting relaxation rate (solid profile) in the $1/T$ region located between the two individual contributions. In order to avoid complications in the drawing the $1/T_2$ plots are omitted. It is useful, however, to recall that under the present conditions $1/T_2$ is dominated by the slowest of the two motions.

(b) When both T_1^{-1} and linewidth values are decreased, it can be concluded that both τ'_c and τ''_c have probably been shortened. The comparison of the relative amounts of these reductions can indicate whether the τ'_c/τ''_c ratio has also been modified, or instead maintained practically unaltered. Cases (a) and (b) apply, respectively, to the relaxation behaviour of the alkyl chains' methylene groups of the phosphatidylcholine/fatty acid and the mixed phosphatidylcholine systems, when the chain length of the additional component is progressively reduced.

The spin-lattice relaxation rate of the $-(CH_2)-_{4-(n-1)}$ groups appear, in fact, to be progressively enhanced and the band linewidth decreased upon gradual reduction of the incorporated fatty acid chain length (from stearic to palmitic to myristic acid). (The selective linewidth reduction, observed only at the level of the $-(CH_2)-_{4-(n-1)}$ resonance band, cannot therefore be attributed to different vesicle sizes.) It should also be noted that, owing to the higher percentage of the esterified vs the free alkyl chains (80 % : 20 %), the T_1 and linewidth values of the chains' groups are essentially due to the host phosphatidylcholine molecules. The results are therefore interpreted on the basis of a progressive decrease of the motional anisotropy of the host phosphatidylcholine chains' proton groups upon incorporation of fatty acids possessing (moderately) shorter chain length. Although the fatty acids are probably located in the hydrophobic core of the bilayer, more or less parallel to the phosphatidylcholine chains [30], significant dynamic perturbations are also induced at the level of the $N^+(CH_3)_3$ groups, as indicated by the decreased T_1 values, observed upon either palmitic or myristic acid incorporation into the bilayer. The result is not surprising; conformational and dynamical perturbations have, in fact, already been observed at the level of the choline groups, as a consequence of packing modifications undergone by the chains (as, for instance, those observed by Levine et al. [31] in phosphatidylcholine vesicles, upon passing through the gel-to-liquid crystal thermal transition).

The interpretation of relaxation times in the mixed phosphatidylcholine bilayers is analogous to the one suggested for the phosphatidylcholine/fatty acid systems, in the sense that by introducing phosphatidylcholines of shorter chain length into distearoyl phosphatidylcholine bilayers, the average motion of the chains' methylene groups are again forced to become more isotropic, as detected by the strongly reduced T_2^{-1} (and $\Delta\nu_{\frac{1}{2}}$) values. On the other hand, the slight but systematic T_1 increments, observed for these groups upon incorporation in the bilayer of dipalmitoyl or dimyristoyl phosphatidylcholine, indicate that the rotational correlation time τ'_c has also been reduced (but to a more minor extent than τ''_c). Inasmuch as T_1 values for the chains' methylene groups observed in distearoyl phosphatidylcholine bilayers are very close to those of dipalmitoyl phosphatidylcholine, while their resonance band linewidth is generally larger for the former, it is suggested that the τ''_c/τ'_c ratio is even larger for distearoyl phosphatidylcholine, as compared with dipalmitoyl phosphatidylcholine. This result may explain why the τ''_c reduction of the chains' methylene groups induced by the shorter chain phosphatidylcholine incorporation into the distearoyl phosphatidylcholine bilayer has little effect on their T_1 relaxation. Significant systematic effect induced in the magnetic relaxation of the chains terminal methyl groups are essentially restricted to their spin-lattice relaxation times. At this time we shall note only that the terminal methyl group motion remains anisotropic in these systems.

These results suggest that Woessner's model can be useful for assessing changes of motional anisotropy of various proton groups in lipid vesicles through T_1 and T_2 relaxation studies. However, it should be explicitly noted that this treatment fails to provide an absolute evaluation of the degree of motional anisotropy. The same intrinsic limitations have been pointed out by Charvolin [32] in deuterium magnetic resonance studies carried out on lyotropic mesophases and attributed to the fact that each motion within these systems modulates several interactions of different origins.

B. Structure of the mixed bimolecular bilayers

A structural model can be proposed for the phosphatidylcholine/fatty acid systems, according to which the carboxyl groups of the free fatty acid chains tend to be located mainly in the region between the carbonyl and the glycerol group of the

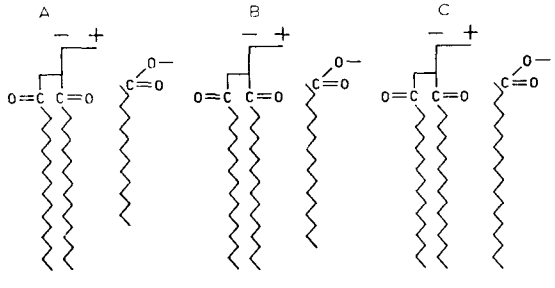

Fig. 5. The diagrams indicate schematically the relative locations of free fatty acids with different chain length (A, myristic; B, palmitic; C, stearic) in a dipalmitoyl phosphatidylcholine monolayer. The "holes" created at the level of the terminal methyl groups in the bilayers containing either palmitic or myristic acid will tend to be partially eliminated through an overall thinning of the hydrocarbon region.

phosphatidylcholine. This location also satisfies the thermodynamic requirements of maintaining hydrophylic groups exposed, to some extent, to the aqueous solvent [2]. According to this model, myristic acid would be preferentially restricted to the outer portion of each dipalmitoyl phosphatidylcholine monolayer, towards the surface, while stearic acid is of sufficient extended chain length to permit its methyl groups to penetrate to the level of the dipalmitoyl phosphatidylcholine terminal CH_3 units (Fig. 5). On the basis of this interpretation very similar magnetic relaxation is expected at the level of the terminal methyl groups in the dipalmitoyl phosphatidylcholine systems containing either palmitic or myristic acid, while the bilayer containing stearic acid should again exhibit a closer similarity with that of pure phosphatidylcholine vesicles, as actually found. Owing to the fluidity of the hydrophobic core, as well as to entropy considerations, the "holes" created around the phosphatidylcholine terminal CH_3 units by the fatty acids of shorter chain will tend to be eliminated through an overall thinning of the bilayer. The structural model suggested for the mixed phosphatidylcholine bimolecular bilayers is strictly analogous. The thinning of the phosphatidylcholine bilayers containing either fatty acids or phosphatidylcholines with shorter chains might in principle occur either (a) via a partial interdigitation of the longer chains at the center of the bilayer or (b) via an increase of the *gauche* isomeric states around the C-C bonds within the longer chains [33]. The lower degree of motional anisotropy found in these systems is in favour of the latter mechanism. It has in fact been pointed out that the conformational dynamics of fatty acyl chains in phosphatidylcholine bilayers is related to the average chain geometry, resulting from a rapid equilibrium between rotational states [33]. In particular, an enhanced average rate of angular fluctuations around the C-C bonds can be related to an increased number of the *gauche* conformations along the chains. The *gauche* isomeric states are expected to occur mostly pairwise along one chain (kink- or jog-like structures [33]) so as to maintain the predominantly parallel packing of the chains within the bilayer. Electron density profile structures, obtained for mixed phosphatidylcholine/fatty acid (Santillan, G. and Blasie, J. K., unpublished) and

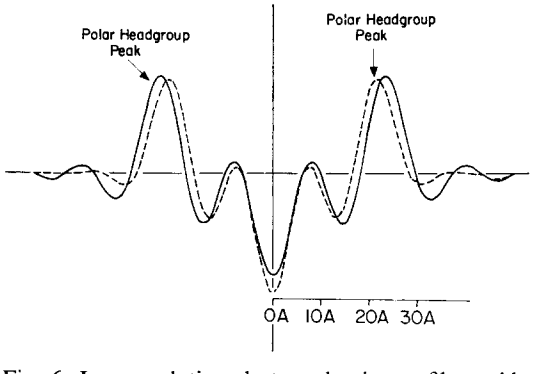

Fig. 6. Low-resolution electron density profiles at identical resolution (13 Å) calculated for distearoyl/dipalmitoyl phosphatidylcholine (—) and distearoyl/dimyristoyl phosphatidylcholine (- - -) bilayers (mol ratio 1 : 1), below the chains' thermal transition. The average polar headgroup separation across the mixed bilayer decreases about 4 Å when dimyristoyl phosphatidylcholine is incorporated into the distearoyl phosphatidylcholine bilayer, as compared with dipalmitoyl phosphatidylcholine incorporation into the distearoyl phosphatidylcholine bilayer, at these molar ratios.

mixed phosphatidylcholine leaflets (Fig. 6) by means of X-ray diffraction below the chains' thermal transition are in agreement with this interpretation (see ref. 34 for complete methods, analysis and interpretation of such low-resolution profile structures). Fig. 6 shows how the incorporation of shorter chain phosphatidylcholines into distearoyl phosphatidylcholine bilayers produces a progressive reduction in the width of the hydrocarbon core of the mixed bilayer without resulting in interdigitation of the longer chain species at the center of the bilayer.

C. Interchain magnetic dipole-dipole interactions

A more complete treatment of proton magnetic relaxation in bilayers would require the consideration of the intermolecular dipole-dipole interactions [35]. By comparing the ^{13}C and ^{1}H T_1 values, measured at 52 °C on fully protonated dipalmitoyl phosphatidylcholine vesicles and on the same phosphatidylcholine highly diluted in a perdeuterio-phosphatidylcholine bilayer, Lee et al. [35] have demonstrated that the intermolecular interactions contribute considerably to the relaxation of the terminal methyl and the choline $N^+(CH_3)_3$ groups.

The spin-lattice relaxation times, alternatively measured on dipalmitoyl phosphatidylcholine vesicles containing either ^{2}H-labeled or unlabeled palmitic acid chains, offer the possibility of isolating the intra- from the inter-molecular average contributions to the spin-lattice relaxation of the phosphatidylcholine chains' proton groups at various temperatures. These contributions can be computed to a first approximation, by means of the equations:

$$\left(\frac{1}{T_1}\right)_{\text{DPPC : PA}} = \left(\frac{1}{T_1}\right)_{\text{intra}} + \left(\frac{1}{T_1}\right)_{\text{inter}} \tag{1}$$

$$\left(\frac{1}{T_1}\right)_{\text{DPPC : [}^2\text{H]PA}} = \left(\frac{1}{T_1}\right)_{\text{intra}} + a\left(\frac{1}{T_1}\right)_{\text{inter}} \tag{2}$$

where DPPC and PA represent dipalmitoyl phosphatidylcholine and palmitic acid, respectively. In Eqn 2, factor a takes into account both the "dilution" of the ^{1}H-chains produced by the [^{2}H]palmitic acid molecules and the deuteron-proton interaction. The values of $(T_1)_{\text{intra}}$ and $(T_1)_{\text{inter}}$ (Table IV), obtained for the system phosphatidylcholine/fatty acid 2 : 1 ($a = 0.81$) suggest (a) that the intermolecular contribution to the longitudinal relaxation is comparable to the intramolecular term around 50 °C, becoming then less and less important as the temperature is further

TABLE IV

INTRA- AND INTERMOLECULAR CONTRIBUTIONS TO THE PROTON SPIN-LATTICE RELAXATION TIMES OF THE CHAINS' $-(CH_2)_{4-15}$ GROUPS IN DIPALMITOYL PHOSPHATIDYLCHOLINE VESICLES CONTAINING FREE PALMITIC ACID CHAINS (LECITHIN: FATTY ACID, 2 : 1)

Temperature (°C)	$(T_1)_{\text{intra}}$ (s)	$(T_1)_{\text{inter}}$ (s)
55	1.09	1.46
66	0.82	4.75
74.5	0.85	6.25

increased above the chains' thermal transition, and (b) that the average correlation time of the intermolecular relative motion ($\tau_{c,\,inter}$) is shorter than the intramolecular rotational correlation time ($\tau_{c,\,intra}$), the latter being of the order of 10^{-9}–10^{-10} s. In fact, in the same temperature range, $(T_1)_{inter}$ increases with temperature, while $(T_1)_{intra}$ goes through a minimum. The fact that $(\tau_{c,\,inter}) < (\tau_{c,\,intra})$ can be explained by considering that the intermolecular relative motion should be thought of mainly as a combination of the intramolecular rotations of the two neighboring chains; diffusion and segmental motion, having longer correlation times, are also expected to give additional but smaller contributions in reducing ($\tau_{c,\,inter}$). In the system phosphatidylcholine/fatty acid 1 : 1 ($a = 0.67$), $(T_1)_{intra}$ decreases from 3.7 to 1.0 s, between 55 and 71.5 °C, while $(T_1)_{inter}$ increases from 0.6 to 3.0 s. In spite of the perturbation of the host phosphatidylcholine's dynamics induced by increasing the relative concentration of incorporated free fatty acid chains, these results are in substantial agreement with those found in the system phosphatidylcholine/fatty acid 2 : 1. The fact that the relaxation times of the choline $N^+(CH_3)_3$ and the chains' terminal $-CH_3$ in these systems are practically the same in the presence of both ^{2}H-labeled and unlabeled palmitic acid (Fig. 2) indicates that no substantial "dilution" effect is effective on the intermolecular proton-proton interactions at the level of these groups, a result that seems to substantiate the models presented in Fig. 5. Further insight on the importance of intermolecular interactions in phosphatidylcholine bilayers at various temperatures will be obtained through a comparison of relaxation times in vesicles of unlabeled dipalmitoyl phosphatidylcholine containing different amounts of ^{2}H-labeled dipalmitoyl phosphatidylcholine.

CONCLUSIONS

The proton magnetic relaxation behavior observed thus far for phosphatidylcholine in single-wall bilayer membranes can be reasonably explained by highly anisotropic motion of the various phosphatidylcholine chain methylene groups. This anisotropic motion arises from a more rapid rotational motion about C-C bond axes and a slower reorientational motion of the C-C bond axes. Such motion would be afforded by coupled *trans-gauche* isomerizations along the chain, which would also maintain the time-averaged orientation of the chains' long axis normal to the bilayer plane as determined by X-ray diffraction studies [36].

The intermolecular contribution to the chains longitudinal relaxation in dipalmitoyl phosphatidylcholine vesicles is comparable to the intramolecular term around 50 °C, becoming then less and less important as the temperature is further increased above the chains' thermal transition.

ACKNOWLEDGEMENTS

We thank Iris Torriani for her help in the T_1 and T_2 measurements on mixed phosphatidylcholine systems. This work was supported by the USPHS grant GM 12202.

REFERENCES

1 Podo, F. and Blasie, J. K. (1975) in MTP International Review of Science, Biochemistry Series, Vol. 2, pp. 97–121 (Fox, C. F., ed.), Butterworths, London
2 McLaughlin, A., Podo, F. and Blasie, J. K. (1973) Biochim. Biophys. Acta 330, 109–121
3 Bloembergen, N., Purcell, G. M. and Pound, R. V. (1948) Phys. Rev. 73, 679–712
4 Kubo, R. and Tomita, K. (1954) J. Chem. Soc. Jap. 9, 888–919
5 Solomon, I. (1955) Phys. Rev. 99, 559–565
6 Seiter, C. H. A. and Chan, S. I. (1973) J. Am. Chem. Soc. 95, 7541–7553
7 Woessner, D. E. (1962) J. Chem. Phys. 36, 1–4
8 Horwitz, A. F., Horsley, W. J. and Klein, M. P. (1972) Proc. Natl. Acad. Sci. U.S. 69, 590–593
9 Podo, F., Cain, J. E. and Blasie, J. K. (1976) Biochim. Biophys. Acta 419, 19–41
10 Podo, F. and Blasie, J. K. (1975) Proc. Natl. Acad. Sci. U.S., in the press
11 Saito, H., Schreier-Muccillo, S. and Smith, I. C. P. (1973) FEBS Lett. 33, 281–285
12 Phillips, M. C., Ladbrooke, B. D. and Chapman, D. (1970) Biochim. Biophys. Acta 196, 35–44
13 Podo, F. and Blasie, J. K. (1974) Proceedings of the Fifth International Symposium on Magnetic Resonance (Bombay), p. 4A9
14 Shimshick, E. J. and McConnell, H. M. (1973) Biochemistry 12, 2351–2360
15 Freeman, R. and Hill, H. D. W. (1971) J. Chem. Phys. 54, 3367–3377
16 Emsley, J. W., Feeney, J. and Sutcliffe, L. H. (1965) High Resolution NMR Spectroscopy, Pergamon Press, Oxford
17 Farrar, T. C. and Becker, E. D. (1971) Pulse and Fourier Transform NMR, Academic Press, New York
18 McDonald, G. G. and Leigh, Jr, J. S. (1973) J. Magn. Resonance 9, 358–362
19 Hahn, E. L. (1950) Phys. Rev. 80, 580–594
20 McLaughlin, A. C., McDonald, G. G. and Leigh, J. S. (1973) J. Magn. Resonance 11, 107–109
21 Lowe, I. J. and Norberg, R. E. (1957) Phys. Rev. 107, 46–61
22 Ernst, R. R. and Anderson, W. A. (1966) Rev. Sci. Instr. 37, 93–102
23 Freeman, R. and Hill, H. D. W. (1971) J. Chem. Phys. 54, 301–313
24 Finer, E. G. (1974) J. Magn. Resonance, 13, 76–86
25 Lichtenberg, D., Petersen, N. O., Girardet, J. L., Kainosho, M., Kroon, P. A., Seiter, C. H. A., Feigenson, G. W. and Chan, S. (1975) Biochim. Biophys. Acta 382, 10–21
26 Kostelnick, R. J. and Castellano, S. M. (1973) J. Magn. Resonance 9, 291–295
27 Lee, A. G., Birdsall, N. J. M., Levine, Y. K. and Metcalfe, J. C. (1972) Biochim. Biophys. Acta 255, 43–56
28 Chan, S. I., Seiter, C. H. A. and Feigenson, G. W. (1972) Biochem. Biophys. Res. Commun. 46, 1488–1492
29 Abragam, A. (1961) Principles of Nuclear Magnetism, p. 292, Oxford University Press, London
30 Kavanau, J. L. (1965) Structure and Function of Biological Membranes, Vol. 1, pp. 74–131, Holden-Day, San Francisco
31 Levine, Y. K., Birdsall, N. J. M., Lee, A. G. and Metcalfe, J. C. (1972) Biochemistry 11, 1416–1421
32 Charvolin, J. (1974) Conference presented at the Vth International Liquid Crystal Conference, Stockholm (June 1974) Adv. Chem., in the press
33 Seelig, A. and Seelig, J. (1974) Biochemistry 13, 4839–4845
34 Lesslauer, W., Cain, J. E. and Blasie, J. K. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1499–1503
35 Lee, A. G., Birdsall, N. J. M. and Metcalfe, J. C. (1973) Biochemistry 12, 1650–1659
36 Cain, J. E., Santillan, G. and Blasie, J. K. (1972) in Membrane Research (Fox, C. F., ed.), pp. 3–12, Academic Press, New York