Biochimica et Biophysica Acta 859 (1986) 171–179
Elsevier

BBA 72926

Mobility of fluorescent probe molecules in lipid bilayer vesicles as studied by steady-state and time-dependent nuclear Overhauser effect measurements in ^{1}H-nuclear magnetic resonance spectroscopy

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(Received November 25th, 1985)

Key words: Fluorescent probe; Lipid bilayer; Nuclear Overhauser effect; ^{1}H-NMR

In order to elucidate the mobilities of the fluorophores of fluorescent 2- and 16-(9-anthroyloxy)palmitic acids (16-AP and 2-AP, respectively) in lipid bilayer vesicles, the steady-state and time-dependent nuclear Overhauser effects in ^{1}H-NMR spectroscopy, but not the fluorescence depolarization in fluorescence spectroscopy, have been measured. The steady-state nuclear Overhauser effect measurements showed an appreciable magnitude of negative nuclear Overhauser effects between the resonances due to the fluorophores of the two fluorescent probes and lipids. These results definitely mean that in lipid bilayers, the fluorophores (anthroyloxy ring) of the fluorescent probes experience other types of motions with much longer correlation times than those detected by the fluorescence depolarization measurements, since at the correlation time showed by the fluorescent method ($1–2 \cdot 10^{-9}$ s or less), no such transfer of the negative nuclear Overhauser effects is expected to occur. The correlation times of the fluorophores, as calculated from the cross-relaxation rates of the anthroyl ring protons of 16-AP and 2-AP, were $3.8 \cdot 10^{-8}$ and $1.1 \cdot 10^{-7}$ s, respectively. These values, respectively, compare favorably with those of the terminal methyl of acyl chains and the choline methyl carbons which were estimated by ^{13}C T_2 relaxation times. Thus, it is concluded that the fluorophores of both 16-AP and 2-AP have a slow form of motion which moves with a similar time scale to those of lipids in addition to the faster one that causes fluorescence depolarization.

Introduction

Fluorescent probes are nowadays extensively used to explore the fluidity and organization of lipids in both synthetic and natural membranes. Analysis of the depolarizing behavior of the fluorescence emitted from the probes in steady-state and/or time-dependent fluorescence anisotropy measurements permits one to obtain information on the dynamics of probe motion which may have sensed the dynamical properties of the surrounding lipids or any other constituents in membranes [1–5]. On account of this importance, the dynamics of probe motion have been extensively studied to inquire into the limitation and usefulness of the probe method [1–6]. Most investigations have been done, of course, by measuring the 'fluorescence' of the probes. It is well known, however, that the time scale which can be monitored by the fluorescent probe method depends on the lifetime of the excited state of the probe molecule used in the experiments. Accordingly, the fluorescence polarization measurements inevitably give a rather limited range for the time scale

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of the motion of a probe itself or that of the surrounding lipids.

Thus, we think that it is necessary to investigate the dynamics of probe motion in lipid bilayers by the other means which can detect motions in a different time scale from that of the fluorescence method. No papers, however, have been published on this subject. Podo et al. [7] investigated the dynamical perturbation of the host lipid molecules induced by incorporation of a probe by ^{1}H-NMR spectroscopy, but not the dynamics of the probe itself. Johns et al. [8] studied the mobility of fluorescent probes by ^{13}C-NMR T_1 relaxation time measurements, but unfortunately this work was done in C^2HCl_3 solutions. Consequently, as a first step to clarify the motional properties of fluorescent probe molecules in lipid bilayers, the present work has been undertaken to investigate the mobility of a fluorophore of a probe molecule in sonicated egg yolk phosphatidylcholine (egg PC) vesicles by steady-state and time-dependent nuclear Overhauser effect measurement in ^{1}H-NMR spectroscopy.

The nuclear Overhauser effect technique has been developed to establish internuclear distances and thus to determine molecular structure in solution [9]. It is also recognized that this technique, especially its time-dependent measurements, are useful to study the mobility of proton pairs in macromolecules having a fixed distance [10–13]. The steady-state nuclear Overhauser effect measurements can also be useful for diagnostic purposes of whether or not a probe molecule in lipid bilayers is moving at a time-constant τ_c which satisfies the condition $\omega^2\tau_c^2 \gg 1$ (ω = the Larmor frequency of the proton) as demonstrated previously for phosphatidylcholine vesicles with incorporated chlorpromazine [14].

The probes chosen in the present study were 2- and 16-(9-anthroyloxy)palmitic acids (2-AP and 16-AP, Fig. 1) respectively, [15–17]. This was because: (1) the locations of the fluorophores (i.e., the anthroyl ring) appear to be settled according to their positions of attachment by an ester linkage with the acyl chain of palmitic acid [7,16,18], (2) the ^{1}H-NMR signals for each proton of the anthroyl ring would be observed as relatively separate signals on account of the presence of an –O–C = O group, and finally (3) the τ_c values in

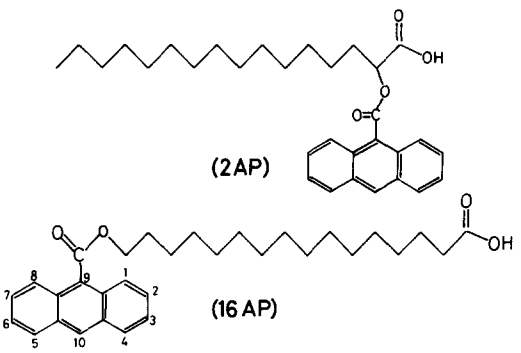

Fig. 1. Structures of 2-AP and 16-AP and numbering scheme for the anthroyl ring.

vesicles (multilayers) of egg PC are available in the literature [19]. A rather high probe/lipid ratio of 1 : 4 was employed, since an accurate determination of the initial rates at which nuclear Overhauser effects develop following a short presaturation time requires a relatively high concentration of the probe solution. The ratio is the same as that employed by Podo et al. [7].

Materials and Methods

Materials. Egg yolk L-α-phosphatidylcholine (egg PC) and L-α-dipalmitoylphosphatidic acid sodium salt (DPPA) were obtained from Sigma. 2- and 16-(9-anthroyloxy)palmitic acids (2-AP and 16-AP, respectively) were purchased from Pharmacia P-L Biochemicals. These materials were used without further purification.

Preparation of vesicles. Single bilayer vesicles were prepared by ultrasonic irradiation of a ^2H_2O suspension of dried egg PC (40 mM) which includes a fluorescent probe (10 mM) and DPPA (0.4 mM). During the sonication, the suspension was cooled in an ice-water bath and bubbled with nitrogen. 1 min of the sonication with 1 min of the interval was repeated 10 times. The vesicle solution was centrifuged at 12000 rpm for 40 min. In order to obtain a similar size of vesicles for both 2-AP and 16-AP, we endeavored to keep these experimental conditions constant. Multi bilayer vesicles were prepared by dispersing the above ^2H_2O suspension of egg PC containing a fluorescent probe and DPPA by a shaking mixer for 2 min. The final concentration of egg PC was 20

mM. No buffer was used and the resulting actual pH values (meter reading) of the solutions were in the range of 4.5–5.5. Aggregation of the probe at the 1 : 4 probe/lipid ratio was checked by ultraviolet absorption spectra. It was found that no aggregation occurred, since no appreciable changes in the ultraviolet absorption spectra were found between the spectra at the ratio of 1 : 4 and those at much lower ratios as in the fluorescence experiments. No deoxygenation was made for these sample solutions before NMR measurements.

Measurements. The ^{1}H-NMR experiments were carried out on a JEOL FX-200 spectrometer operating at 200 MHz. Ambient probe temperatures were 23.3–23.8°C. All the spectra were taken under conditions of 45° pulse flip angle and 8 K of memory for 2000-Hz spectral width. Steady-state and time-dependent nuclear Overhauser effect difference spectra were obtained as described previously [14]. The time-dependent nuclear Overhauser effects were measured by a truncated driven type of experiment [20]. The strength of the irradiation radiofrequency field (Hz) was ca. 5 Hz ($\gamma \mathbf{H}_2/2\pi$). This magnitude does not belong to the limit of high-power irradiation which can afford an accurate cross-relaxation rate [13]; however, it can give a good approximate value, minimizing saturation of nearby protons. Chemical shifts were referenced to internal ^{2}HHO.

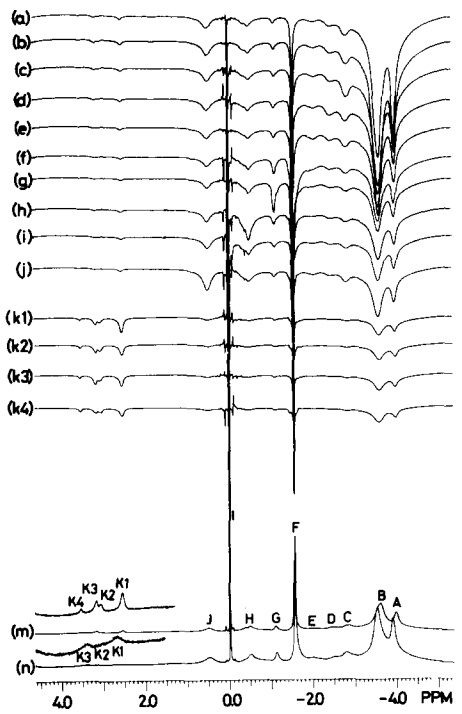

Fig. 2. (a–k4) Steady-state nuclear Overhauser effect difference spectra of 16-AP-egg PC vesicles in ^2H_2O; (m) ^{1}H-NMR spectrum of 16-AP-egg PC vesicles in ^2H_2O; (n) ^{1}H-NMR spectrum of 2-AP-egg PC vesicles in ^2H_2O. In a–k4, each spectrum was obtained by irradiating the corresponding peak labeled A–K4 in m.

Results

Fig. 2 shows the steady-state nuclear Overhauser effect difference spectra (a–k4) of 16-AP-egg PC vesicles in ^2H_2O and normal ^{1}H-NMR spectra of 16-AP-egg PC (m) and 2-AP-egg PC (n) vesicles in ^2H_2O. The steady-state nuclear Overhauser effect difference spectra were obtained by irradiating the peaks A–K4 labeled in spectrum m for 5 s. Assignments for these peaks are summarized in Table I together with their chemical shifts. The assignments for peaks due to egg PC were taken from those of Hauser et al. [21]; those due to the anthroyl ring protons were determined by assuming the same order of chemical shifts as in the $CDCl_3$ solutions of 16-AP and 2-AP. In the $CDCl_3$ solutions, resonances due to the anthroyl ring protons could easily be assigned by spin-decoupling and nuclear Overhauser effect experiments. The numbering in the anthroyl ring is shown in Fig. 1. Some of the steady-state nuclear Overhauser effect values (%) are summarized in Table II.

Locations of the anthroyl rings of 16-AP and 2-AP are considered to be near the center of a lipid bilayer and the polar head group, respectively [7,16,18]. The present data, of chemical shifts support this view; for example, peaks A, B and C in the 16-AP-egg PC solution resonated in a higher field than the corresponding peaks in the 2-AP-egg PC solution (see Fig. 2 m and 2 n, or Table I), whereas the reversed situations were noted with peaks D, F, G and H.

Fig. 3a, b show typical examples of the time-dependent nuclear Overhauser effect difference spectra of 16-AP- and 2-AP-egg PC vesicles in ^2H_2O where, in both cases, peak K1 at about 2.6 ppm was irradiated, and the irradiation time was varied

TABLE I

PEAK ASSIGNMENTS AND THEIR CHEMICAL SHIFTS OF 16-AP-EGG PC AND 2-AP-EGG PC VESICLES IN ^2H_2O

Peak	Assignment	Chemical shifts [a]	
		16-AP-egg PC	2-AP-egg PC
A	CH_3(PC, 2-AP)	−3.99	−3.90
B	$(CH_2)_n$(PC, 16-AP, 2-AP)	−3.61	−3.53
C	CH_2–C = C, CH_2–CHOCO(2-AP)	−2.82	−2.77
D	CH_2CO(PC, 16-AP)	−2.43	−2.47
E	= C–CH_2–C =	−2.00	−1.98
F	$N^+(CH_3)_3$	−1.54	−1.56
G	CH_2N^+	−1.10	−1.12
H	CH_2OCO, CH_2OP, CH_2O(16-AP)	−0.48	−0.49
I	^{2}HHO (solvent)	0.00	0.00
J	CH = CH, CHOCO(PC, 2-AP)	0.51	0.49
K1 [b]	H-2,3,6,7(16-AP, 2-AP), H-4(2-AP) [c]	2.55	2.70
K2 [b]	H-4,5(16-AP), H-5(2-AP) [c]	3.07	3.06
K3 [b]	H-1,8(16-AP, 2-AP), H-10(2-AP)	3.17	3.41
K4 [b]	H-10(16-AP)	3.55	

[a] In ppm, ±0.01 ppm for peaks A–J, and ±0.02 ppm for peaks K1–K4.
[b] Anthroyl ring protons.
[c] Assignments for H-4 and H-5 in peaks K1 and K2 can be interchanged.

from 100 ms to 5 s. The time-dependence of nuclear Overhauser effects for a two-spin system is predicted by Eqn. 1,

$$\eta_i(t) = (\sigma_{ij}/\rho_i)(1 - e^{-\rho_i t}) \quad (1)$$

where $\eta_i(t)$ is the nuclear Overhauser effect at a time t, σ_{ij} is the cross-relaxation rate between the observed proton(s) i and the irradiated proton(s) j, and ρ_i is the direct relaxation rate of i [9]. Since the initial gradient of $\eta_i(t)$ is given in Eqn. 2,

$$(d\eta_i(t)/dt)_0 = \sigma_{ij} \quad (2)$$

σ_{ij} can be obtained either from the least-squares fit of the observed $\eta_i(t)$ from Eqn. 1 or more roughly obtained from an nuclear Overhauser effect value at a very short time.

TABLE II

VALUES (%) OF STEADY-STATE NUCLEAR OVERHAUSER EFFECTS, $\eta(5)$, FOR 16-AP-EGG PC AND 2-AP-EGG PC VESICLES IN ^2H_2O

Error limits are ±3% for peaks A, B and F, and ±5% for peaks G, H, J and K1.

Peak observed	Peak irradiated					
	F: $N^+(CH_3)_3$		A: CH_3		K1: anthroyl ring [a]	
	16-AP	2-AP	16-AP	2-AP	16-AP	2-AP
A	−40	−32			−8	−14
B	−46	−35			−8	−13
F			−20	−19	−4	−7
G			−23	−21	−4	−7
H	−40	−32	−32	−34	−7	−11
J	−57	−39	−50	−51	−11	−14
K1	−25	−28	−30	−46		

[a] H-2,3,6,7 in 16-AP and H-2,3,4,6,7 in 2-AP.

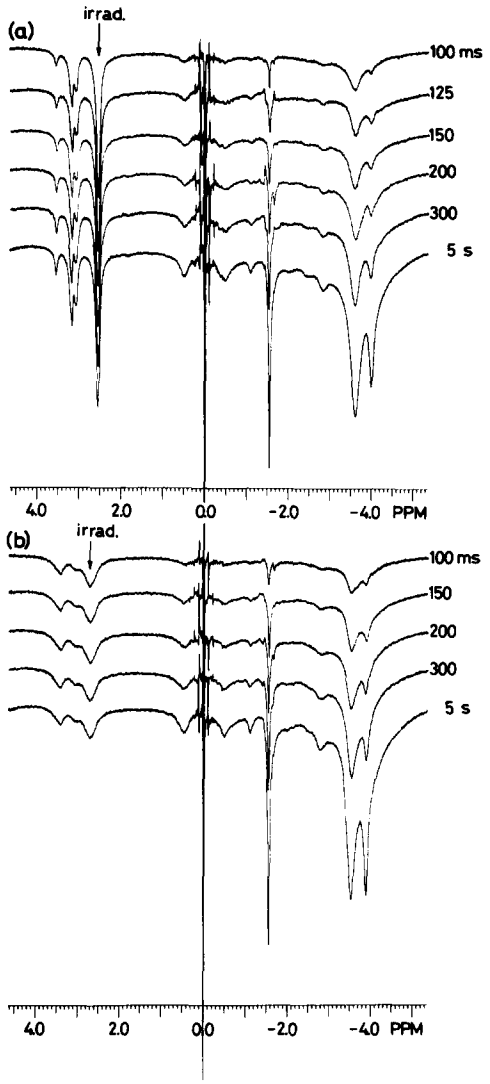

Fig. 3. Time-dependent nuclear Overhauser effect difference spectra of (a) 16-AP- and (b) 2-AP-egg PC vesicles in ^2H_2O; the irradiated peak (K1) is shown by an arrow. Preirradiation times are indicated on the right.

Fig. 4 shows a buildup curve of nuclear Overhauser effect for peak K4 of 16-AP, where the ordinate indicates the percentage of growth of these effects relative to the steady-state value ($\eta(5) = -0.732$). The line is a least-squares fit to Eqn. 1. Besides peak K1, we also irradiated peaks K2, K3, and K4, respectively, in order to check the internal consistency of σ_{ij} within the same anthroyl ring. Cross-relaxation rates of 16-AP thus

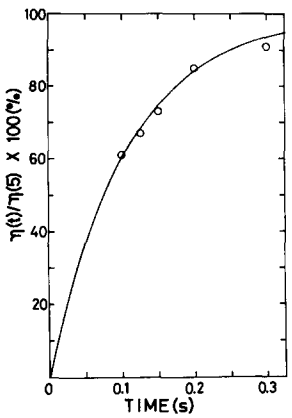

Fig. 4. Plot of $\eta(t)/\eta(5)\times 100$ (%) vs. preirradiation time for peak K4 of Fig. 3a, where peak K1 was irradiated. $\eta(5) = -0.732$. The line is a least-squares fit to Eqn. 1.

obtained are summarized in Table III.

Table III informs us that σ_{ij} values between adjacent proton pairs, the internuclear vector of which is inclined 30° to the long axis of the anthroyl ring, are -8.6 to -8.9 s^{-1}, whereas those parallel to this axis are -7.5 to -7.7 s^{-1}. This difference in σ_{ij} with respect to the different types of proton pairs is reasonable, because the long axis of the anthroyl ring lies parallel to the bilayer normal [22]; hence, a rotational motion around this axis does not contribute to the dipole-dipole interactions between protons lying parallel to the axis. Table III also shows that σ_{ij} between protons which are not adjacent to each other further decreases with increasing distance. This may be a result of attenuated cross-saturation owing to the mediated proton(s). Since such a decreasing order of magnitude of σ_{ij} is physically acceptable, we may conclude that the σ_{ij} values shown in Table III are reasonable.

On the other hand, as shown in Fig. 3b, the nuclear Overhauser effect value of peak K3 of 2-AP had already reached its steady-state value even at a preirradiation time of 100 ms. Thus, we obtained σ_{ij} for 2-AP from an initial slope of the nuclear Overhauser effect at a shorter time. A very short irradiation time of 17.5 ms was employed to give a nuclear Overhauser effect value of -0.43 ± 0.07 for peak K3. From this value, the σ_{ij} of 2-AP was determined as -24.6 ± 4 s^{-1}.

The literature values of correlation times for

TABLE III

CROSS-RELAXATION RATES (s^{-1}) IN ANTHROYL RING PROTONS OF 16-AP-EGG PC VESICLES IN ^2H_2O

Data are given as standard deviation ($\pm$S.D.) in the least-squares fit of the observed $\eta_i(t)$ to Eqn. 1.

Peaks irradiated	Assignment [a]	Peak observed			
		K1	K2	K3	K4
K1	H-2,3,6,7	0	-8.9 ± 0.4	-8.6 ± 0.3	-6.7 ± 0.2
K2, K3	H-4,5,1,8	-8.9 ± 0.1	0	0	-7.5 ± 0.3
K4	H-10	-2.8 ± 0.1	-7.7 ± 0.2	-2.7 ± 0.1	0

[a] The numbering in the anthroyl ring is shown in Fig. 1.

the fluorescent probes are those in the multilayer vesicles [19]. Accordingly, we tried to measure the time-dependent nuclear Overhauser effects for multilayer vesicles. In the multilayer vesicles, the peaks due to the probe were much broader than those in the sonicated single bilayer vesicles; therefore, we could follow time-dependent nuclear Overhauser effects only for 16-AP by raising the temperature of its ^2H_2O solution to 85°C to sharpen the peaks as much as possible. At this temperature, the linewidths of the peaks due to the anthroyl ring protons were similar to those shown in sonicated 2-AP-egg PC vesicles (Fig. 2n). A peak at about 3.09 ppm, which originates from five or six protons of H-(1),2,3,4,5,6, was irradiated and a peak at about 3.67 ppm, which originates from two or three protons of H-(1),8,10, was observed. The least-squares fit to the observed nuclear Overhauser effect values at the irradiation times of 15 ($\eta = -0.33$), 30 ($\eta = -0.51$), and 45 ms ($\eta = -0.58$) gave a value of -31.4 ± 0.8 s^{-1} as a cross-relaxation rate.

Time-dependent nuclear Overhauser effects for 16-AP and 2-AP in $CDCl_3$ solutions (15–20 mM) have been measured by varying the irradiation times from 300 ms to 5 s in order to obtain correlation times for the fluorophores not embedded in the lipid bilayers. In the 16-AP solution, the peak due to H-1,4,5,8 was irradiated and that due to H-10 was observed ($\eta(5) = 0.206$). In the 2-AP solution, the peak due to H-4,5 was irradiated and that due to H-10 was observed

TABLE IV

CROSS-RELAXATION RATES σ_{ij} (s^{-1}) AND CORRELATION TIMES τ_c AND τ_f (s) FOR ANTHROYL RING OF FLUORESCENT DYES (16-AP, 2-AP AND 2-AS)

NOE, Nuclear Overhauser effect. n.d., not determined.

Method	Systems	Parameters	Fluorescent dyes	
			16-AP	2-AP/2-AS [a]
^{1}H-NMR, NOE	sonicated egg PC	σ_{ij}	-8.9 ± 0.1	-24.6 ± 4
		τ_c	$3.8\pm0.1\cdot10^{-8}$	$1.1\pm0.3\cdot10^{-7}$
	multilayer egg PC	σ_{ij}	-31.4 ± 0.8	n.d.
	85°C	τ_c	$1.35\pm0.03\cdot10^{-7}$	n.d.
	23°C [b]	τ_c	$3.36\pm0.07\cdot10^{-7}$	
^{1}H-NMR, NOE	C^2HCl_3 solution	σ_{ij}	0.048 ± 0.003	0.170 ± 0.008
		τ_c	$7.7\pm0.1\cdot10^{-10}$	$4.5\pm0.3\cdot10^{-10}$
Fluorescence [a]	multilayer egg PC	τ_f	$1.0\cdot10^{-9}$	$2.2\cdot10^{-9}$

[a] Taken from Ref. 19. $\tau_f = 1/6R$, R = rotational rate.

[b] The τ_c at 85°C was converted into that at 23°C by assuming an activation energy of 3.1 kcal/mol in the Arrhenius equation.

($\eta(5) = 0.232$). The cross-relaxation rates obtained are compared in Table IV with those in lipid bilayers.

From these σ_{ij}'s, correlation times, τ_c's, were determined (Table IV). The τ_c's are isotropic rotational correlation times and were calculated according to Eqn. 3,

$$\sigma_{ij} = \left(\gamma^4 \cdot \hbar^2/10 r_{ij}^6\right)\left(6\tau_c/\left(1+4\omega^2\tau_c^2\right) - \tau_c\right) \quad (3)$$

where γ is the magnetogyric ratio of the proton, $\hbar$ is Planck's constant divided by 2π, and r_{ij} is the interproton distance. The r_{ij} was assumed to be 2.5 Å; this is a typical distance between adjacent protons in an aromatic ring. The τ_c's obtained in the present method can be considered to be reflecting the mobility of each fluorophore in lipid bilayers and not to be reflecting the overall rotation of a vesicle on the order of approx. 10^{-6} s (t_R), since the τ_R of 10^{-6} s gives the σ_{ij} values as large as -233 s^{-1} for the protons separated by 2.5 Å, indicating the significant mobility of the fluorophore itself in the vesicle. The calculated τ_c values in C^2HCl_3 solutions were comparable to those obtained by Johns et al. [8] for anthroyl ring carbon atoms of 6-AS (S, stearic acid) at a state of inverted micelle-like aggregates of the probe in C^2HCl_3 ($4.2 \cdot 10^{-10}$ s). In the case of 16-AP in multilayer vesicles, the τ_c at 23°C was tentatively calculated by assuming an activation energy of 3.1 kcal/mol [23] in the Arrhenius equation. Table IV also includes the correlation times, τ_f's, for 16-AP and 2-AS in multilayer vesicles which were determined by the fluorescence polarization experiments with an excitation wavelength of 384 nm ($\tau_f = 1/6R$, R = rotational rate [19]; the method for obtaining the R value is that described by Lakowicz et al. [24], which employs both steady-state anisotropy and differential phase measurements. These τ_f's are comparable to those determined by Vincent et al. [25,26] for 16-AP ($0.8–1.3 \cdot 10^{-9}$ s) and 2-AS ($4.5 \cdot 10^{-9}$ s) in dipalmitoyl-phosphatidylcholine vesicles at 47°C. We stress that these τ_c's and τ_f's are 'isotropic' rotational correlation times; therefore, discussions based on exact comparison among these values would not be justified, since in lipid bilayers the motion of a fluorophore may not be isotropic, but anisotropic. Thus, in the following section, we will discuss the mobility of fluorescent probe molecules by considering τ_c's and τ_f's as a measure of order of magnitude of a correlation time of the fluorophore.

Discussion

The degree of fluorescence depolarization depends on the rotation of the fluorophore relative to the direction of its emission transition moment [1–5]. In the case of *n*-(9-anthroyloxy) fatty acid probes, the emission transition moment is considered to lie in the plane of the anthracene ring and has an angle of about 30° to the absorption moment which is parallel to the short axis of the anthracene ring; both their acyl chains and the long axis of the ring align parallel to the bilayer normal [18,22]. However, recently, Vincent et al. have shown that at the excitation wavelength of 381 nm [25,26], which is nearly an identical wavelength to that used by Kutchai et al. [19], the absorption transition moment is parallel to the long axis of the ring. In either case, if the excitation wavelength at about 380 nm was employed, both in-plane and out-of-plane modes of rotation of the anthroyl ring, which may be related to the motion of the acyl chain of a fatty acid probe, contribute to the depolarization [25,26]. The average correlation times (τ_f) for these modes of 16-AP and 2-AS in multilayers are $1.0 \cdot 10^{-9}$ and $2.2 \cdot 10^{-9}$ s, respectively [19]. On the other hand, on the basis of proton T_1 studies in ^{1}H-NMR spectroscopy, Chan et al. [27–29] have shown that the correlation times for the reorientation ($\tau_\perp$) and rotational isomerization ($\tau_\parallel$) of the acyl chains of lipids are, respectively, ca. 10^{-7} s and 10^{-9}–10^{-10} s for multilayers and 10^{-8}–10^{-9} s and 10^{-10}–10^{-11} s for sonicated single bilayers. Although the τ_f values are in the range of $\tau_\parallel$ values for multilayers, they are much shorter than $\tau_\perp$. Johns et al. [8] have suggested from ^{13}C-NMR studies that the anthroyl ring and the carbon atom to which it is attached move as one unit, the type of motion being determined by the motion of the acyl carbon atom. Thus, if we consider the structural resemblance between the fatty acid probes and lipids, we may expect that there must be a form of motion which moves slowly at the order of the time constant $\tau_\perp$. The question now arises

whether the anthroyl ring is moving independently from the motion of the surrounding lipids at the time constant indicated by the fluorescence method or is there another motion which moves more slowly than the rate shown by the time constant τ_f. The presently obtained steady-state nuclear Overhauser effect difference spectra clearly reply to this question. For example, Fig. 2 (or Table II) shows that 16-AP is incorporated into the lipid bilayer and that it is moving at the same time scale as those of vesicles and/or of lipid motions in membranes. This is easily verified by noting that irradiation for peaks arising from egg PC caused negative nuclear Overhauser effects for anthroyl ring proton resonances (spectra a–j) and vice versa for the peaks K1–K4 (spectra k1–k4). The same sort of findings was also observed for 2-AP-egg PC vesicles (Table II). In the sonicated egg PC vesicles, if there are no such slow motions in both the egg PC and probe molecules that satisfy the condition $\omega^2\tau_c^2 \gg 1$, no transfer of negative nuclear Overhauser effects is expected to occur [14,30,31]. The τ_c's determined in sonicated bilayer vesicles were $3.8 \cdot 10^{-8}$ (16-AP) and $1.1 \cdot 10^{-7}$ s (2-AP) and were much longer than the corresponding τ_f values. In multilayers, the τ_c increases further as expected (Table IV). These values fully satisfy the above condition. The τ_c values of 16-AP in single and multi bilayers are roughly comparable to those of the corresponding $\tau_\perp$ values; however, the τ_c of 2-AP was much longer than $\tau_\perp$. This may arise from the fact that the fluorophore of 2-AP resides at a polar head group where molecular motion is highly constrained. The differences in linewidths of the peaks due to the anthroyl ring protons between 16-AP and 2-AP solutions clearly reflect the differences in mobility of the fluorophore in lipid bilayers, since in C^2HCl_3 solutions, the τ_c values of the anthroyl rings were similar to each other (Table IV).

Interestingly, the magnitudes of τ_c of 16-AP and 2-AP are comparable, respectively, to those of terminal methyl ($5.8 \cdot 10^{-8}$ s) and choline methyl carbons ($1.0 \cdot 10^{-7}$ s) which were estimated from ^{13}C-NMR linewidth (T_2) measurements for sonicated egg PC vesicles at 50°C [23]. The correlation times from ^{13}C T_2 relaxation times characterize the slow motions of acyl chains as in the nuclear Overhauser effects (spin diffusion) measurements in the present study. Thus, it can be concluded that (i) the fluorophores of both 16-AP and 2-AP have modes of motions which move more slowly than the time constant shown by the fluorescent probe method and that (ii) their mobilities are similar to that of a lipid motion in bilayers, that is, they are moving cooperatively with the lipids; this is especially so for the case of 16-AP.

Quite recently, Lakowicz et al. [32] presented a new method for obtaining time-resolved fluorescence anisotropies by multi-frequency phase-modulation technique. They analyzed the anisotropies of diphenylhexatriene and perylene in lipid bilayers by a two- or three-correlation time model and found that one of the correlation times becomes much longer than the value obtained by a usual single correlation time model or by a hindered rotator model. It would be of interest to carry out similar experiments and analysis procedures with 16-AP and 2-AP in lipid bilayers. In the two- or three-correlation time model, the magnitude of one of the correlation times should be comparable to those of our values which were determined by the nuclear Overhauser effect method.

Acknowledgement

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Japan.

References

1 Radda, G.K. (1975) Methods Membrane Biol. 4, 97–188
2 Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394
3 Lakowicz, J.R. (1980) J. Biochem. Biophys. Methods 2, 91–119
4 Kinosita, K., Jr., Kawato, S. and Ikegami, A. (1977) Biophys. J. 20, 289–305
5 Kawato, S., Kinosita, K., Jr. and Ikegami, A. (1977) Biochemistry 16, 2319–2324
6 Sklar, L.A. (1983) in Membrane Fluidity (Kates, M. and Manson, L.A., eds.), pp. 99–131, Plenum Press, New York
7 Podo, F. and Blasie, J.K. (1977) Proc. Natl. Acad. Sci. USA 74, 1032–1036
8 Johns, S.R., Willing, R.I., Thulborn, K.R. and Sawyer, W.H. (1979) Chem. Phys. Lipids 24, 11–16
9 Noggle, J.H. and Schirmer, R.E. (1971) The Nuclear Over-

hauser Effect: Chemical Applications, Academic Press, New York
10 Clore, G.M. and Gronenborn, A.M. (1984) FEBS Lett. 172, 219–225
11 Ramaprasad, S., Johnson, R.D. and La Mar, G.N. (1984) J. Am. Chem. Soc. 106, 3632–3635
12 Olejniczak, E.T., Poulsen, F.M. and Dobson, C.M. (1981) J. Am. Chem. Soc. 103, 6574–6580
13 Dobson, C.M., Olejniczak, E.T., Poulsen, F.M. and Ratcliffe, R.G. (1982) J. Magn. Reson. 48, 97–110
14 Kuroda, Y. and Kitamura, K. (1984) J. Am. Chem. Soc. 106, 1–6
15 Cadenhead, D.A., Kellner, B.M.J., Jacobson, K. and Papahadjopoulos, D. (1977) Biochemistry 16, 5386–5392
16 Thulborn, K.R. and Sawyer, W.H. (1978) Biochim. Biophys. Acta 511, 125–140
17 Tilley, L., Thulborn, K.R. and Sawyer, W.H. (1979) J. Biol. Chem. 254, 2592–2594
18 Thulborn, K.R., Tilley, L., Sawyer, W.H. and Treloar, F.E. (1979) Biochim. Biophys. Acta 558, 166–178
19 Kutchai, H., Chandler, L.H. and Zavoico, G.B. (1983) Biochim. Biophys. Acta 736, 137–149
20 Wagner, G. and Wüthrich, K. (1979) J. Magn. Reson. 33, 675–680
21 Hauser, H., Phillips, M.C., Levine, B.A. and Williams, R.J.P. (1975) Eur. J. Biochem. 58, 133–144
22 Badley, R.A., Martin, W.G. and Schneider, H. (1973) Biochemistry 12, 268–275
23 Gent, M.P.N. and Prestegard, J.H. (1977) J. Magn. Reson. 25, 243–262
24 Lakowicz, J.R., Prendergast, F.G. and Hogen, D. (1979) Biochemistry 18, 508–519
25 Vincent M., de Foresta, B., Gallay, J. and Alfsen, A. (1982) Biochemistry 21, 708–716
26 Vincent, M., de Foresta, B., Galley, J. and Alfsen, A. (1982) Biochem. Biophys. Res. Commun. 107, 914–921
27 Petersen, N.O. and Chan, S.I. (1977) Biochemistry 16, 2657–2667
28 Feigenson, G.W. and Chan, S.I. (1974) J. Am. Chem. Soc. 96, 1312–1319
29 Seiter, C.H.A. and Chan, S.I. (1973) J. Am. Chem. Soc. 95, 7541–7553
30 Akasaka, K. (1979) J. Magn. Reson. 36, 135–140
31 Kalk, A. and Berendsen, H.J.C. (1976) J. Magn. Reson. 24, 343–366
32 Lakowicz, J.R., Cherek, H. and Maliwal, B.P. (1985) Biochemistry 24, 376–383