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EMISSION-WAVELENGTH-DEPENDENT DECAY OF THE FLUORESCENT PROBE *N*-PHENYL-1-NAPHTHYLAMINE

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We have measured the fluorescence decay of *N*-phenyl-1-naphthylamine using the phase-modulation method, in several solvent systems and egg phosphatidylcholine vesicles. The decay is monoexponential in pure solvents (both polar and non-polar) of low viscosity. In polar viscous solvents or in non-polar solvents containing an added polar solute, the decay is heterogeneous and emission wavelength dependent. In such cases, dielectric relaxation and/or excited-state complexing give rise to a shift of the emission spectrum on the nanosecond time scale. Emission-wavelength-dependent decay was also observed when *N*-phenyl-1-naphthylamine was bound to egg phosphatidylcholine vesicles. From these results as well as the position of the emission spectral maximum, we conclude that *N*-phenyl-1-naphthylamine probes the ester-carbonyl region of the phospholipid acyl chains, where it undergoes an excited-state reaction. This result contradicts the often made assumption that *N*-phenyl-1-naphthylamine probes the deeper hydrocarbon region of the bilayer.

Since the time of their introduction by Weber and Laurence in 1954 [1], *N*-arylaminonaphthalene derivatives have been applied to a variety of problems in protein and membrane structure [2,3]. Their usefulness derives from the marked solvent sensitivity of their fluorescence parameters. Although a number of aspects of their spectroscopic properties remain to be elucidated, these probes seem to exhibit an increased dipole moment in the first excited state [4–12], and form excited-state complexes with polar solutes [8,13–15].

The uncharged derivative *N*-phenyl-1-naphthylamine has been widely used to probe the hydrophobic region of membranes [3,8,16]. In the present work * the nanosecond decay of *N*-phenyl-1-naphthylamine has been examined when the probe is dissolved in several solvent systems, as well as bound to egg lecithin vesicles. It is shown that under certain conditions the decay kinetics are emission wavelength dependent. These results have important implications for the use of this probe in membrane studies.

Materials and Methods

N-Phenyl-1-naphthylamine was purchased from Eastman Kodak (Rochester, NY) and recrystallized from spectroscopic-grade methanol. The recrystallized *N*-phenyl-1-naphthylamine was shown to be free of fluorescent impurities by showing that its emission spectrum was independent of exciting wavelength, and by the homogeneity of its fluorescence decay in various solvents (see Results). Paraffin oil (Uvasol grade) from E. Merck (Darmstadt), and triolein obtained from Nu-Check Prep, Inc. (Elysian, MN) were used without further purification. Egg lecithin was obtained from two sources, both of which yielded identical results. It was initially prepared from hen eggs by the method of Litman [17], and shown to be

* Presented in preliminary form at the Biophysical Society Meeting in Atlanta, GA [21].

greater than 99% pure by thin-layer chromatography. Phosphatidylcholine was subsequently obtained from Makor Chemicals (Jerusalem, Israel), and its purity was also found to be greater than 99%. Cyclohexane (spectro grade) and decane (Gold Label) were produdcts of Aldrich (Milwaukee, WI), and glycerol (spectro grade) and methanol (spectro grade) were obtained from Fisher Scientific (Pittsburgh, PA). All other solvents and chemicals were of the highest purity commercially available.

Small unilamellar phosphatidylcholine vesicles were prepared by sonication of lipid dispersions using the method of Huang and Thompson [18]. Lipid which had been stored at –20°C in chloroform or ethanol was lyophilized and suspended in buffer (20 mM Tris-HCl/50 mM NaCl, pH 7.4) at approx. 30 mM phospholipid. This suspension was sonicated for 1 h at 4°C under nitrogen using a Branson sonicator set at about 70 W. Titanium particles were removed by centrifugation in a Sorvall SS-34 rotor at 17 000 rev./min for 45 min, and the vesicles were stored under nitrogen at 0–4°C. The vesicles were not further chromatographed (e.g., on Sepharose 4B) and are therefore a mixture of small (approx. 200 Å d) vesicles and a small amount (probably less than 5%) of larger multilamellar liposomes. Lipid concentration was determined as inorganic phosphate by the method of Gomori [19].

To incorporate N-phenyl-1-naphthylamine into vesicles, microliter quantities of a concentrated stock solution of N-phenyl-1-naphthylamine in methanol were added while vortexing a 1–3 mM (phospholipid) suspension of vesicles; the final concentration of methanol in the labeled suspension was kept below 0.2% (v/v). Essentially all of the probe is bound to the vesicles with the lipid to probe ratios at which these measurements were performed (over 200 : 1). N-Phenyl-1-naphthylamine was dissolved in triolein and paraffin oil by dilution from a 0.15 M stock solution in decane.

Fluorescence measurements

Fluorescence lifetimes were determined by the phase-modulation technique, in an instrument built by SLM Instruments (Urbana, IL); steady-state polarizations were measured in the same apparatus. Monochromators were used for both excitation and emission in all measurements. Details of the fluorescence methods have been presented elsewhere [20,42], and here we will briefly review the salient features of the method.

In a homogeneous system with single exponential decay, the lifetimes determined under sinusoidal excitation by either phase delay (τ_p) or relative modulation (τ_m) are equal at all modulation frequencies. When the emission (for excitation by a pulse) is described by a sum of exponentials,

$$I(t) = \sum_i A_i \exp(-t/\tau_i) \tag{1}$$

under sinusoidal excitation the observed τ_p and τ_m differ and are given by:

$$\tau_p = w^{-1} \tan \Phi \tag{2}$$

$$\tau_m = w^{-1}(M_r^{-2} - 1)^{1/2} \tag{3}$$

$$\tan \Phi = \frac{\sum_{i=1}^{n} \alpha_i \sin \varphi_i \cos \varphi_i}{\sum_{i=1}^{n} \alpha_i \cos \varphi_i} \tag{4}$$

$$M_r^2 = \left(\sum_{i=1}^{n} \alpha_i \sin \varphi_i \cos \varphi_i\right)^2 + \left(\sum_{i=1}^{n} \alpha_i \cos^2 \varphi_i\right)^2 \tag{5}$$

in which $w = 2\pi f$ (f = modulation frequency), Φ and M_r are the observed phase angle and relative modulation, φ_i is the phase angle of the ith component with fluorescence lifetime τ_i ($\tau_i = w^{-1} \tan \varphi_i$), and α_i is the fractional steady-state intensity ($0 \leqslant \alpha_i \leqslant 1$) due to the ith component ($\Sigma\alpha_i = 1$). The α_i terms in Eqns. 4 and 5 are related to the A_i in Eqn. 1 by $\alpha_i = A_i\tau_i / \Sigma_i A_i \tau_i$. When the $A_i > 0$ in Eqn. 1, the following conditions hold: (a) $\tau_m > \tau_p$ at all frequencies; (b) τ_m and τ_p increase with decreasing frequency. When an excited-state reaction or relaxation process is present and new species or intermediates are created which emit at red-shifted wavelengths, the decay kinetics will vary across the emission spectrum, with the mean decay time increasing with increasing wavelength. In such cases, A_i in Eqn. 1 may in some cases take on negative values, and when observed by phase modula-

tion fluorimetry, the restrictions (a) and (b) above no longer hold and some α_i may now be negative (with $\Sigma_i \alpha_i = 1$). The observation of the breakdown of ordinary heterogeneity restrictions on τ_m and τ_p alone is sufficient (but not necessary) to demonstrate the presence of an excited-state reaction. In particular, the failure of (a) and (b) to hold is expected at observation wavelengths on the red side of the emission spectrum, because emission from the newly created state(s) dominates in this region.

The τ_m and τ_p data, obtained at two or three modulation frequencies, was analyzed for two or three exponential components by a procedure in which x^2, defined by

$$x^2 = \sum_w \left\{ \left(\frac{\tau_p^t - \tau_p}{\sigma_p} \right)^2 + \left(\frac{\tau_m^t - \tau_m}{\sigma_m} \right)^2 \right\} \quad (6)$$

is minimized by varying the α_i and φ_i. τ_p^t and τ_m^t are the theoretical phase and modulation lifetimes calculated according to Eqns. 2–5, σ_p and σ_m are the estimated standard deviations of the measured lifetimes, and the sum is performed over all modulation frequencies at which data were obtained.

Fluorescence emission and excitation spectra were taken on a Perkin-Elmer MPF-2A fluorometer, and data given in this paper concerning emission wavelengths are uncorrected for the wavelength-dependent response of the instrument. Absorption data were obtained on a Cary 210 spectrophotometer.

Results

Solvent studies

When dissolved in pure decane, cyclohexane, ethanol or methanol, the fluorescence decay of *N*-phenyl-1-naphthylamine at 20°C is monoexponential and independent of emission wavelengths. The lifetimes, measured at 10–20 nm intervals across the emission band, were found to be: decane, 4.1 ± 0.1 ns (360–460 nm); cyclohexane, 4.0 ± 0.1 ns (365–440 nm); ethanol, 6.5 ± 0.1 ns, methanol, 5.5 ± 0.1 ns (365–440 nm). In viscous polar solvents such as glycerol, however, *N*-phenyl-1-naphthylamine exhibits an emission-wavelength-dependent decay (Matayoshi, E.D., unpublished data), as was observed for the phenylnaphthylamine derivatives 1-analinonaphthalene-8-sulfonate [5], 2-(*p*-toluidono)naphthalene-6-sulfonate [9], and *N*-phenyl-1-naphthylamine [12]. The heterogeneous decay in these cases has been interpreted as arising from the relaxation of polar solvent molecules surrounding the probe. Time-dependent spectral shifts (or, equivalently, an increase in mean decay time with increasing emission wavelength) are obtained because emission will occur from one of a series of partially solvated excited states. The increased lifetimes are associated with lower energies of emission (red-shifted spectra) because the latter arise from the later intermediates in the solvation process.

We investigated the emissive properties of *N*-phenyl-1-naphthylamine in two viscous non-polar solvents, triolein and paraffin oil. These solvents were chosen as isotropic analogues to the hydrophobic region of egg lecithin bilayers. As shown in Fig. 1A, the observed fluorescence lifetime of *N*-phenyl-1-naphthylamine dissolved in triolein is a function of emission wavelength. The mean decay time, as measured by τ_m at a modulating frequency of 30 MHz, increases with increasing wavelength. The change in τ_m between 370 and 460 nm is about 2 ns at 0°C, but is reduced to only 0.6 ns at 40°C. Phase lifetimes at 30 MHz (not shown) were also measured and found to increase with wavelength, and exhibit the condition $\tau_p < \tau_m$ on the blue side, but $\tau_p > \tau_m$ on the red side. As discussed under Methods, the latter anomalous condition arises from an excited-state reaction.

Fig. 1B displays the steady-state polarization values, p, of *N*-phenyl-1-naphthylamine in triolein, corresponding to the above conditions. As expected from the lifetime measurements, the polarization decreases monotonically from maximal values at the blue end of the emission band. At all three temperatures, the value of p at 480 nm is nearly 20% lower than at 370 nm.

It should be pointed out that while the observed decay times are independent of the excitation wavelength, the polarization is not. Excitation-polarization data are given in Fig. 1c, which demonstrates that the broad low energy absorption spectrum of *N*-phenyl-1-naphthylamine obscures an absorption band due to a higher energy transition. The marked decrease in p with decreasing excitation wavelength is in contrast to the constancy of p observed with *N*-phenyl-2-naphthylamine derivatives [22]. These observations

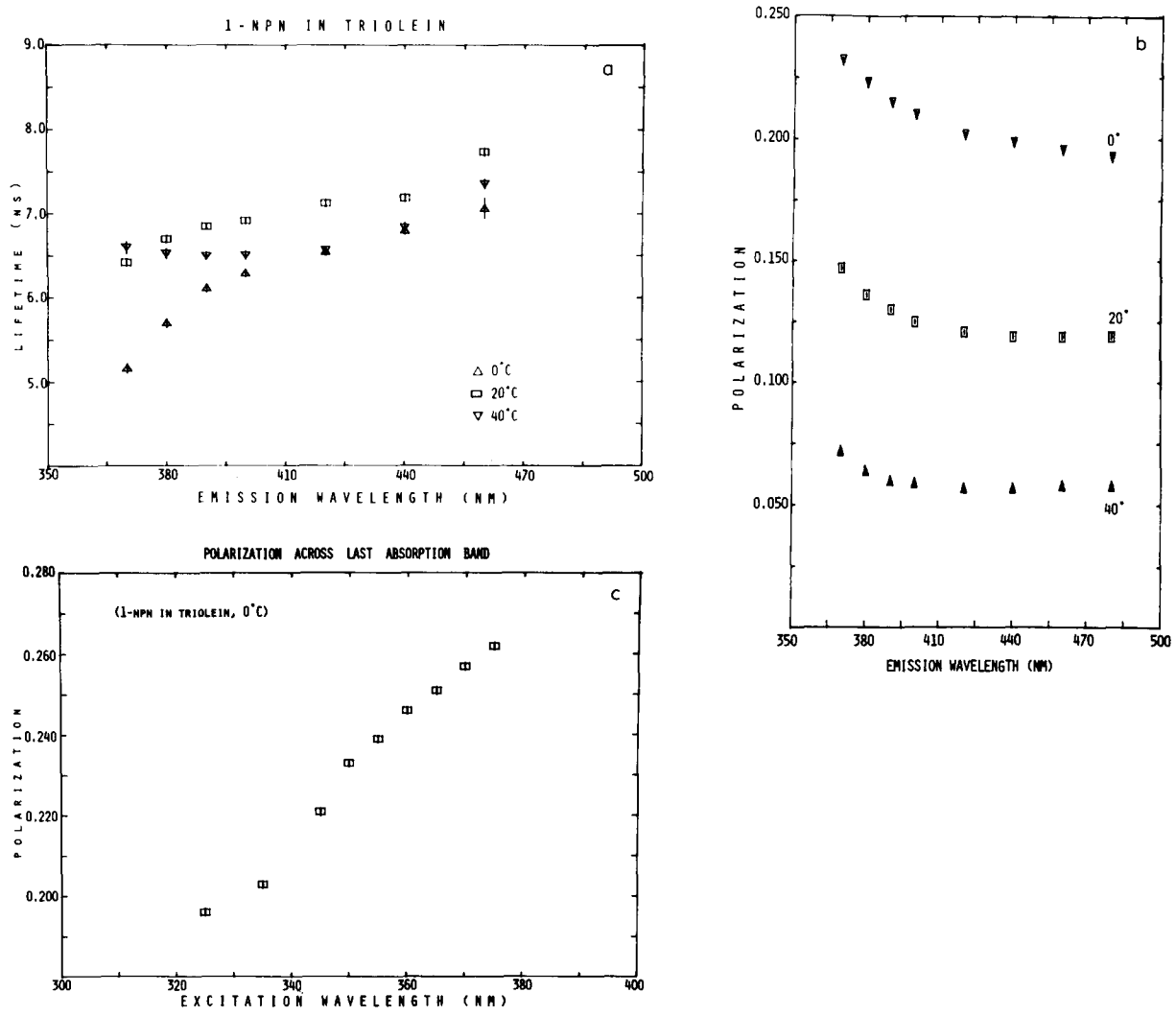

Fig. 1. (a) Modulation lifetimes (30 MHz) as a function of emission wavelength for 10 μM *N*-phenyl-1-naphthylamine in triolein. Excitation = 355 nm, 1 nm bandpass, emission bandpass = 8 nm. The standard deviation is shown by the vertical bars. (b) Steady-state polarization of *N*-phenyl-1-naphthylamine in triolein as a function of emission wavelength. Excitation = 355 nm, except for the 0°C data, for which excitation = 340 nm. Excitation and emission bandpasses were 8 nm each. (c) Polarization of *N*-phenyl-1-naphthylamine across the lowest energy-absorption band. The sample used was 10 μM *N*-phenyl-1-naphthylamine in triolein. Emission was monitored at 400 nm, 8 nm bandpass; excitation bandpass was 4 nm.

are consistent with molecular orbital calculations [4], from which it is predicted that the polarization of the two lowest energy transitions is very different for *N*-phenyl-1-naphthylamine, but similar for *N*-phenyl-2-naphthylamine.

In contrast to the time-dependent spectral shift of *N*-phenyl-1-naphthylamine occurring in triolein, measurements of *N*-phenyl-1-naphthylamine dissolved in paraffin oil indicate that both p and τ are independent of emission wavelength (Fig. 2a and 2b, rectangles). The equality of τ_m and τ_p at 30 MHz also indicates that the decay kinetics are monoexponential, with $\tau = 4.7 \pm 0.1$ ns at 10°C. These results imply that the excited-state spectral shifts obtained in the latter

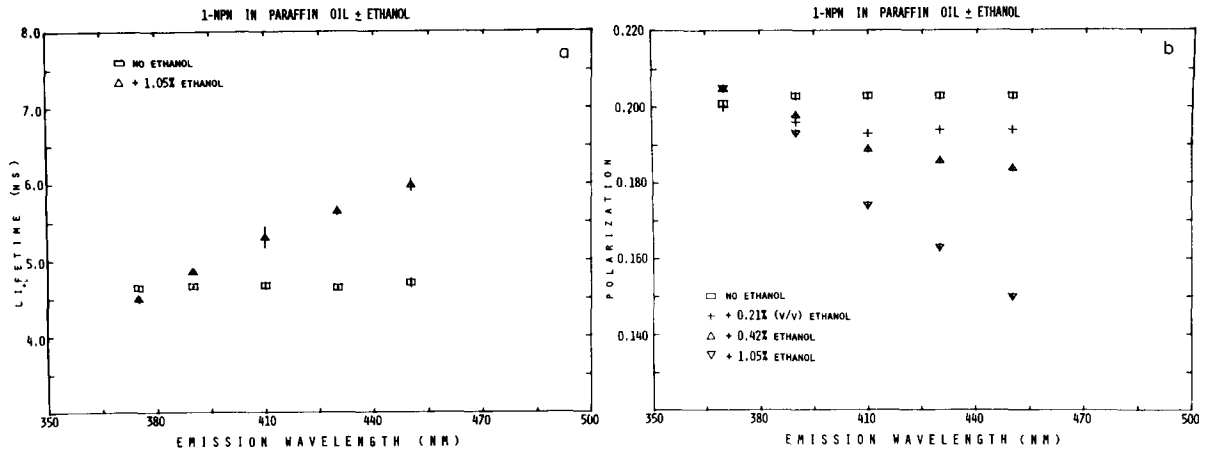

Fig. 2. Emission of *N*-phenyl-1-naphthylamine (20 μM) in paraffin oil, 10°C. (a) Modulation lifetimes (30 MHz) as a function of emission wavelength, with and without 1% (v/v) ethanol. Excitation = 350 nm, emission bandpass was 8 nm. (b) Steady-state polarization as a function of emission wavelength, in the presence of 0–1% ethanol. Excitation = 350 nm, excitation and emission bandpass were 8 nm each.

two systems cannot be due to a viscous constraint on intramolecular relaxation. Since the only significant difference between triolein and paraffin oil lies in the ester dipole of the former, it is inferred that either dielectric relaxation or exciplex formation is responsible for the time-dependent emission in triolein.

An interesting test of the sensitivity of *N*-phenyl-1-naphthylamine to these solvent/solute interactions is to observe the effects of low (submolar) concentrations of polar molecules on its decay in non-polar solvents (Refs. 23–25). We performed such experiments with *N*-phenyl-1-naphthylamine in paraffin oil and cyclohexane. In confirmation of the results of Bhaumik and Hardwick [13], the addition of alcohols such as ethanol was found to cause a red-shift of the steady-state emission spectrum. The addition of 0.5% (v/v) ethanol to a cyclohexane solution of *N*-phenyl-1-naphthylamine resulted in a 17 nm red-shift of the emission spectrum but left the absorption spectrum unaffected. We also observed that the addition of increasing amounts of ethanol (up to 1% v/v) to paraffin oil or cyclohexane caused the fluorescence decay to become wavelength-dependent. In a viscous medium such as paraffin oil, the increase in mean decay time with increasing emission wavelength (Fig. 2a) is most simply demonstrated by the parallel inverse changes in the steady-state polarization, which decreases with wavelength. As shown in Fig. 2b, the polarization becomes increasingly wavelength-dependent with increasing ethanol concentration.

As was noted above, the emission of *N*-phenyl-1-naphthylamine in both ethanol and paraffin oil is monoexponential and no spectral relaxation is detected. The emission maximum in ethanol is some 40 nm red-shifted from the peak in cyclohexane. While the stoichiometry of *N*-phenyl-1-naphthylamine/ethanol exciplexes is unknown, the apparent difference between the excited-state reaction occurring in dilute versus pure ethanol solution is presumably due to stoichiometry. In the pure solvent, the ethanolic shell around the fluorophore is saturated immediately following excitation and all decay occurs from the completely relaxed state. However, in dilute solution, infrequent collisions between excited *N*-phenyl-1-naphthylamine and ethanol would give rise to emission from both complexes and uncomplexed fluorescent states.

In Table I the results are presented for two frequency lifetime measurements of *N*-phenyl-1-naphthylamine dissolved in cyclohexane containing 0.5% v/v (= 0.086 M) ethanol. The measured values of both τ_m and τ_p at each frequency increase monotonically with increasing wavelength. The heterogeneity analysis indicated that the assumption of two components yields good fits at nearly all wavelengths. Although the long component (τ_2) is essentially con-

TABLE I

WAVELENGTH DEPENDENT DECAY OF *N*-PHENYL-1-NAPTHYLAMINE IN CYCLOHEXANE/ETHANOL

Emission wavelength (nm)	Observed lifetimes (ns)				Heterogeneity analysis				
	30 MHz		18 MHz		α_1	τ_1	α_2	τ_2	x^2
	τ_m	τ_p	τ_m	τ_p					
365	3.23	2.50	3.67	2.68	0.8 ± 0.1	2.2 ± 0.2	0.2 ± 0.1	8.3 ± 0.7	0.03
375	3.39	2.72	3.84	2.99	0.8 ± 0.1	2.4 ± 0.2	0.2 ± 0.2	7.4 ± 0.3	0.43
385	3.85	3.12	4.28	3.42	0.8 ± 0.2	2.5 ± 0.2	0.2 ± 0.2	7.3 ± 0.3	0.06
395	4.41	3.81	4.83	4.13	0.8 ± 0.2	3.3 ± 0.2	0.2 ± 0.2	8.0 ± 0.4	0.31
410	5.00	4.79	5.29	4.99	0.8 ± 0.2	4.3 ± 0.4	0.2 ± 0.2	9.0 ± 1.0	1.30
425	5.30	5.40	5.55	5.57	1.0 ± 0.2	5.5 ± 0.2	0.0 ± 0.2	10.0 ± 3.0	6.30
440	5.52	5.88	5.68	5.91	1.2 ± 0.2	6.0 ± 0.2	−0.2 ± 0.2	8.0 ± 0.5	6.50

stant at 7.6 ns throughout, the value of τ_1 increases with wavelength. These results are not consistent with a simple two-state reaction, which would yield τ_1 and τ_2 independent of, but α_1 and α_2 dependent on, wavelength. Since, as suggested by the small x^2 values of Table I, a two component fit is a good approximation to the decay, fluorescence appears to be occurring from a series of increasingly red-shifted intermediates (corresponding to τ_1). In the simplest interpretation, τ_1 at the blue-most wavelengths corresponds to the lifetime of uncomplexed *N*-phenyl-1-naphthylamine in cyclohexane, shortened from the lifetime of 4 ns in pure cyclohexane because of the additional collisional quenching taking place. The formation of multiple intermediates would arise if a sequential series of equilibria were occurring between exciplexes of differing stoichiometries. However, the short lifetime of *N*-phenyl-1-naphthylamine probably precludes a time-dependent diffusion mechanism. We can only surmise that in addition to the excited-state reaction with ethanol, heterogeneous ground-state complexes (i.e., of different ethanol numbering) might also contribute to the observed decay. (The lack of an effect on the absorption spectrum does not support this hypothesis, but as noted below, the solvent-induced shifts of *N*-phenyl-1-naphthylamine absorption do not lend themselves easily to interpretation.) Such ground-state heterogeneity would give rise to ordinary decay heterogeneity superimposed on that due to the excited-state reaction.

The final state, represented by $\tau_2 \approx 7.6$ ns, must have a spectral distribution and intensity such that it contributes appreciably to the observed intensity at all wavelengths. Although τ_2 is longer than the lifetime observed in pure ethanol or any other solvents at room temperature, it is unclear whether one can easily extrapolate the pure-solvent behavior of *N*-phenyl-1-naphthylamine to its emissive characteristics in a heterogeneous solvent shell. Beyond 425 nm, the observed τ_p values become longer than τ_m, and it becomes necessary to assign a negative amplitude to one component for a reasonable fit. This result, and the monotonic increase in mean decay time with wavelength, demonstrate unambiguously that red-shifted intermediates are formed in the excited state. The poorer fits on the red side undoubtedly result from heterogeneity analysis difficulties encountered when apparent near-homogeneity is observed, resulting in $\alpha_1 \to 1$.

Vesicle studies

When *N*-phenyl-1-naphthylamine was incorporated into egg lecithin vesicles, its fluorescence decay was found to be heterogeneous and to vary as a function of emission wavelength. As shown in Fig. 3a, the mean decay time (as measured by τ_m at 30 MHz) increases monotonically with wavelength across the emission spectrum at 0, 20 and 40°C. The corresponding decrease in the steady-state polarization with increasing wavelength, under the same conditions, is shown in Fig. 3b. A full set of τ_m and τ_p data, measured at 30 and 18 MHz, is given in Table II. These data can be summarized as follows: (1) the decay function at all wavelengths cannot be ade-

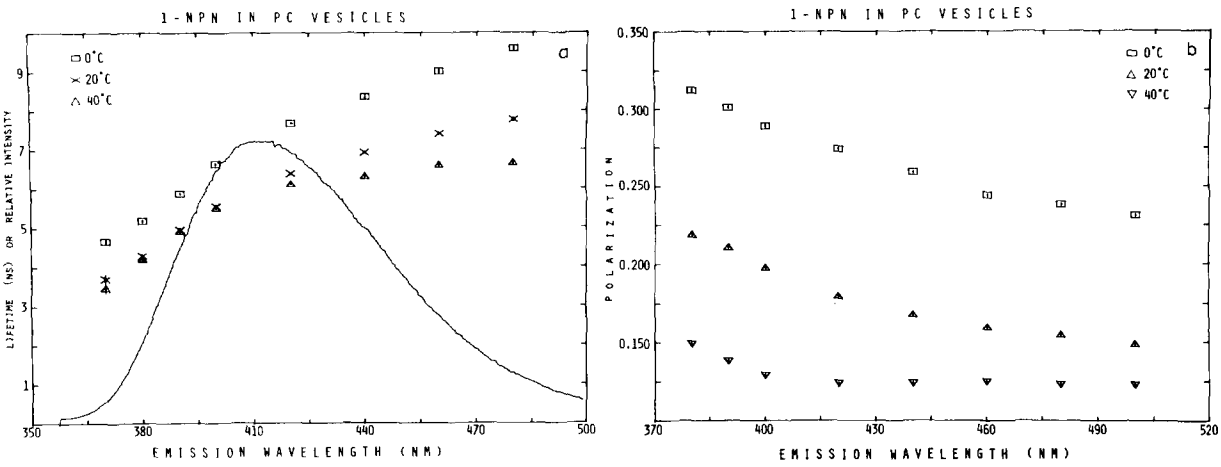

Fig. 3. Emission of *N*-phenyl-1-naphthylamine bound to egg lecithin vesicles at 0, 20, and 40°C. (a) Modulation lifetimes (30 MHz) as a function of emission wavelength. Excitation = 355 nm, emission bandpass was 8 nm. Sample contained 2.5 mM lipid, 10 μM probe. Also shown (solid curve) is the technical emission spectrum of *N*-phenyl-1-naphthylamine bound to vesicles, (b) steady-state polarization as a function of emission wavelength. Excitation = 355 nm; excitation and emission bandpass were 8 nm each. Sample contained 1 mM lipid, 4 μM probe. The error in p, shown by the vertical bars, is ±0.001.

quately described as either the sum or the difference of two exponentials (all two-component fits yielded chi-square values corresponding to $P \ll 0.01$); (2) at wavelengths below 450–460 nm, $\tau_p < \tau_m$, but at longer wavelengths, $\tau_p > \tau_m$; (3) τ_m (30) $> \tau_m$ (18) at all wavelengths.

TABLE II

LIFETIME DATA OF *N*-PHENYL-1-NAPHTHYLAMINE BOUND TO EGG LECITHIN VESICLES

Values are ns

Emission wavelength (nm)	30 MHz		18 MHz	
	τ_m	τ_p	τ_m	τ_p
380	5.9 ± 0.2	3.7 ± 0.4	5.7 ± 0.3	4.3 ± 0.4
390	6.7 ± 0.1	4.7 ± 0.2	6.5 ± 0.3	5.1 ± 0.4
400	7.4 ± 0.1	5.7 ± 0.2	6.9 ± 0.1	5.9 ± 0.3
410	8.0 ± 0.1	6.4 ± 0.3	7.4 ± 0.2	6.4 ± 0.3
420	8.4 ± 0.1	7.3 ± 0.2	7.9 ± 0.2	7.1 ± 0.3
430	8.8 ± 0.1	8.1 ± 0.2	8.2 ± 0.2	7.6 ± 0.3
440	9.2 ± 0.1	9.0 ± 0.3	8.7 ± 0.2	8.1 ± 0.4
450	9.6 ± 0.1	9.7 ± 0.4	8.8 ± 0.3	8.4 ± 0.5
460	9.9 ± 0.1	10.9 ± 0.8	9.1 ± 0.3	9.1 ± 0.7
470	10.1 ± 0.1	12.0 ± 1.0	9.2 ± 0.3	9.2 ± 0.6
480	10.3 ± 0.1	12.3 ± 1.0	9.5 ± 0.3	9.9 ± 0.8
490	10.8 ± 0.3	12.9 ± 1.4	9.3 ± 0.4	9.9 ± 0.8

Heterogeneity of probe environment must of course be considered when dealing with a non-covalent probe bound to a membrane. Such heterogeneity may indeed contribute to the decay complexity which is evident in this system. However, the increase in decay times across the emission band and the anomalous conditions $\tau_p > \tau_m$ and τ_m (30) $> \tau_m$ (18), when taken together, conclusively demonstrate the occurrence of a time-dependent spectral shift resulting from an excited-state reaction.

Discussion

It has been established from studies of the emission of *N*-phenyl-1-naphthylamine in various solvents [8,13] and from molecular orbital calculations [4] that *N*-phenyl-1-naphthylamine undergoes a large increase in dipole moment upon excitation. The well-documented time-dependent spectral shifts of 1-anilinonaphthalene-8-sulfonate and 2-(*p*-toluidono)naphthalene-6-sulfonate at low temperatures and in viscous polar solvents [5,9] can therefore be explained in terms of solvent dielectric relaxation. Similarly, it was demonstrated that when bound to phosphatidylcholine vesicles, the probe (2-(*p*-toluidono)naphthalene-6-sulfonate was susceptible to spectral shifts occurring on the nanosecond time scale, which were attributed to dielectric relaxation at the polar mem-

brane surface [9–11]. For this reason, we were initially surprised to find that *N*-phenyl-1-naphthylamine, usually assumed to sample the hydrophobic interior of the bilayer [3,16], also exhibited complex, wavelength-dependent decay.

An examination of the literature indicates that, apart from the fact that *N*-phenyl-1-naphthylamine is uncharged, there are two experiments which suggest a hydrophobic location for the probe. In both cases a comparison is made with the charged analogue 1-anilinonaphthalene-8-sulfonate, whose location in the bilayer is known from X-ray diffraction [26,27] and nuclear magnetic resonance [28,29] to be near the membrane/water interface. In one experiment, H_2O was replaced by ^2H_2O, and the fluorescence of membrane-bound 1-anilinonaphthalene-8-sulfonate was observed to be enhanced nearly 2-fold, whereas the fluorescence of *N*-phenyl-1-naphthylamine showed no enhancement [3]. It is not, however, known how far and how many water molecules actually penetrate into the bilayer. Furthermore, the detailed physical mechanism of the solvent isotope effect is not understood [30]. Therefore, from this study it can only be concluded that *N*-phenyl-1-naphthylamine is more shielded from the aqueous phase than 1-anilinonaphthalene-8-sulfonate, but not necessarily located deep in the hydrophobic region.

The question of probe location was also dealt with in the proton nuclear magnetic resonance studies on phosphatidylcholine vesicles by Colley and Metcalfe [31]. The addition of 1-anilinonaphthalene-8-sulfonate to vesicles was observed to produce very large shifts in the resonance of the phospholipid choline protons, but *N*-phenyl-1-naphthylamine produced only very small shifts. The conclusion that *N*-phenyl-1-naphthylamine is located in a region which is farther from the choline methyls than 1-anilinonaphthalene-8-sulfonate is again very qualitative, because the ring current effects involved are only effective within approx. 8 Å and will depend on the relative alignment of the interacting groups.

Perhaps a more revealing indication of *N*-phenyl-1-naphthylamine's bilayer location can be obtained from the position of its emission spectrum. For example, in fluid solvents at room temperature, the emission maximum ranges from 375 nm (decane, cyclohexane), to 415 nm (ethanol) 419 nm (methanol), 422 nm (*N,N*-dimethylformamide), and 442 nm (formamide). A highly blue-shifted spectrum cannot be used as evidence for a non-polar probe environment unless relaxation effects (solvent or probe) can be ruled out. Incomplete relaxation of either solvent dipoles around the excited probe, or of probe excited-state geometry, would impose a blue-shifting of the emission from the relaxed case. However, when *N*-phenyl-1-naphthylamine is bound to phosphatidylcholine vesicles, its emission peak at 417 nm is red-shifted with respect to pure hydrocarbon solvents, which strongly suggests that the probe is solvated by polar groups in the first excited state, equilibrium geometry.

In principle, the absorption maximum could be used as an indication of *N*-phenyl-1-naphthylamine's bilayer location in the ground state and first excited state, Frank-Condon geometry. A red-shifting of the absorption maximum with increasing solvent polarity or polarizability is predicted when the chromophore undergoes an increase in dipole moment in the first excited state [32]. We have in fact observed that the absorption is blue-shifted in non-polar solvents such as decane and cyclohexane (λ_{max} = 337 nm) and red-shifted in polar solvents such as dimethyl formamide (λ_{max} = 342.5 nm). The 342 nm λ_{max} observed in phosphatidylcholine vesicles, would, therefore, seem to support a more polar location of *N*-phenyl-1-naphthylamine. However, additional studies (data not shown) performed, for example, on a homologous series of alcohols indicate numerous deviations from a simple correlation of the absorption maximum with either polarity or polarizability and we conclude that the absorption data cannot be used reliably to characterize the environment of *N*-phenyl-1-naphthylamine.

What then is the mechanism by which the nanosecond spectral shift is exhibited by *N*-phenyl-1-naphthylamine bound to the membrane? In the work discussed in this paper, the limited number of frequencies at which data were taken has prevented us from fitting the kinetics of the fluorescence decay to theoretical models other than a simple two-state reaction. However, it is clear that the following excited-state effects must be considered: (1) intramolecular relaxation; (2) an excited-state reaction with bilayer dissolved water; (3) probe/probe intermolecular interactions; (4) vertical diffusion of the probe during the excited state; and (5) an excited-state reaction with the phospholipid ester carbonyl groups. We will now

consider each of these possibilities.

(1) The constancy and homogeneity of the decay across the emission spectrum of *N*-phenyl-1-naphthylamine dissolved in paraffin oil is of considerable interest since it implies, unlike the case for the 9-anthroyloxy fatty acids [21,42], that microviscous constraints on the intramolecular relaxation of the probe in the excited state are insignificant. That *N*-phenyl-1-naphthylamine undergoes a change in nuclear geometry in the excited state (in non-polar as well as polar solvents) is implied by the large Stokes' shift of the emission spectrum, and also by the difference between the experimental radiative lifetime (τ_0) and the theoretical radiative lifetime (τ_0^t). For *N*-phenyl-1-naphthylamine in hexane, for example, these lifetimes differ by a factor $(\tau_0^t/\tau_0) = 2.5$ [8]. Molecular rigidity and the degree of planarity have frequently been postulated as major factors determining the sensitivity of the emissive parameters of anilinonaphthalene derivatives [1,22,33–37]. The dual fluorescence of *N,N*-dialkylaniline derivatives can be explained on the basis of intramolecular relaxation to a charge-transfer state [43]. Our results do not resolve the question of whether intramolecular relaxation between two or more emitting states occurs in a polar environment. Molecular orbital calculations on *N*-phenyl-1-naphthylamine [4], however, indicate that the energy of emission is rather insensitive to the dihedral angle between the phenyl and naphthalene rings. This result indicates that relaxation between two different intramolecular geometries, for example, is not likely to constitute the source of the time-dependent spectral shifts observed in triolein or phosphatidylcholine vesicles.

(2) *N*-Phenyl-1-naphthylamine might conceivably undergo an excited-state reaction with water molecules dissolved in the bilayer. Of course, the ^2H_2O experiment of Radda and Vanderkooi [3] (described above) implies that *N*-phenyl-1-naphthylamine is shielded from such interactions with water. However, it could be argued that an excited reaction occurs but no isotope-induced fluorescence enhancement is effected. If this were the case, the decay kinetics of *N*-phenyl-1-naphthylamine in phosphatidylcholine vesicles might be expected to differ in the presence of ^2H_2O vs. H_2O. We have not carried out this experiment, and therefore cannot absolutely rule out this possibility.

(3) Intermolecular excited-state interactions between *N*-phenyl-1-naphthylamine molecules in phosphatidylcholine vesicles can be ruled out on the basis of the absence of a dependence of the fluorescence polarization, lifetime, or spectrum on *N*-phenyl-1-naphthylamine/lipid mole ratio (Kleinfeld, A.M. and Solomon, A.K., unpublished data).

(4) Vertical diffusion of *N*-phenyl-1-naphthylamine towards the membrane surface, i.e., through a polarity gradient, could give rise to time-dependent emission spectra which are increasingly red-shifted in time. However, it is difficult to rationalize the source for such directed diffusion. A diffusion mechanism is also unlikely because of the rates involved. While the diffusion parameters of *N*-phenyl-1-naphthylamine are unknown, Vanderkooi and Callis [38] have found that the lateral diffusion coefficient of pyrene in egg lecithin is only $3 \cdot 10^{-8}$ cm^2/s, and Traüble and Sackmann [39] obtained a value of $1 \cdot 10^{-8}$ cm^2/s for a spin-labeled androstane. Vertical diffusion towards the membrane surface is expected, if anything, to be slower, and even for $D \approx 3 \cdot 10^{-8}$ cm^2/s, *N*-phenyl-1-naphthylamine will move less than 3 Å within its lifetime of approx. 5 ns.

(5) The results in triolein clearly indicate that the presence of the polar carbonyl group of the fatty acyl chains is by itself sufficient to give rise to nanosecond spectral shifts. Our lifetime data do not resolve the question of whether the shift is due to exciplex formation, dielectric relaxation, or both processes. But the comparison between triolein and paraffin oil, along with the position of the emission maximum in phosphatidylcholine vesicles and the four points discussed above, strongly suggests that the nanosecond spectral shifts in phosphatidylcholine vesicles are a consequence of *N*-phenyl-1-naphthylamine's location near the ester-carbonyl groups of the bilayer. This result contradicts the often-made assumption that *N*-phenyl-1-naphthylamine probes the deeper hydrocarbon region of the bilayer.

Investigations similar to those reported in this paper but using *N*-phenyl-2-naphthylamine have been reported by DeToma and Brand [15] and Badea et al. [12]. These authors measured the time-resolved emission spectra to determine the kinetics of the relaxation process itself, and compared this with the decay of the fluorescence anisotropy. In agreement with the results for *N*-phenyl-1-naphthylamine, these authors

found that the decay of *N*-phenyl-2-naphthylamine is emission-wavelength-dependent in polar solvents and liposomes. They also suggest, from a comparison of the relaxation rate and decay anisotropy, that the decay kinetics in liposomes are probably due to interactions with the polar carbonyl group of the phospholipid glycerol backbone and not to dissolved water. There are, however, some significant differences between the results of our study and theirs. Although we find, in agreement with DeToma and Brand [15], that the decay of *N*-phenyl-1-naphthylamine is emission-wavelength-independent in cyclohexane and ethanol (they obtain $\tau = 4.6$ ns and $\tau = 7.3$ ns for cyclohexane and ethanol, respectively) our results in cyclohexane plus 0.1 M ethanol are substantially different from theirs. In particular these authors found that: (1) both components are wavelength-independent ($\tau_1 \approx 5.8$ ns and $\tau_2 \approx 1.8$ ns); (2) the short component is associated with a progressively more negative α_2 value; (3) α_2 is negative at wavelengths above 395 nm (almost 30 nm to the blue of the *N*-phenyl-1-naphthylamine case) and; (4) the values of $[\alpha_2]$ at the red side of the spectrum are substantially greater than our values.

In addition, Badea et al. [12] found that the decay of *N*-phenyl-2-naphthylamine in liposomes is similar to that in glycerol, which can be reasonably well described by the Bakshiev formulation [40,41]. According to this scheme, the overall decay can be separated into two parts: a wavelength-independent decay, and a spectral relaxation contour which characterizes the dielectric relaxation process. Their results indicate that negative components occur only at the red side of the spectrum, whereas our results suggest negative α_2 values even at the blue-most wavelengths. While the reason for these differences is unclear, as we have noted above (see the results of the excitation wavelength dependence of the polarization), the structural differences between *N*-phenyl-1-naphthylamine and *N*-phenyl-2-naphthylamine can result in substantial spectroscopic differences. It may also be that these structural differences can affect the excited-state reactivity with the structurally restricted region around the carbonyl groups of the glycerol backbone.

As this and other work demonstrates, the fluorescence decay of membrane-bound probes such as *N*-phenyl-1-naphthylamine, *N*-phenyl-2-naphthylamine [12], and the 9-anthroyloxy fatty acids [21], are non-exponential. The results discussed in this paper clearly emphasize that any quantitative application of *N*-phenyl-1-naphthylamine, in which a one-term lifetime is assumed, may be precluded by the intrinsically complex heterogeneity and wavelength dependence of the emission which obtains under certain conditions. It is also clear that, instrumental difficulties aside, there are theoretical aspects of the photophysical mechanism of *N*-phenyl-1-naphthylamine fluorescence which must be elucidated before the observed decay can be analyzed in terms of physically meaningful parameters, thereby permitting a molecular characterization of the probe's environment. Hence, when a better understanding of the excited state behavior of *N*-phenyl-1-naphthylamine is attained, it is likely to provide for a far more sensitive probe of membrane structure.

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