Study of Heterogeneous Emission of Parinaric Acid Isomers Using Multifrequency Phase Fluorometry†

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ABSTRACT: The fluorescence lifetimes of parinaric acid (PnA) isomers have been measured in pure solvents and in synthetic phospholipid membranes over a wide temperature range. In pure solvents, dodecane, ethanol, and cyclohexanol, the emission is well described by three exponential components of approximately 40, 12, and 2 ns. The preexponential factor of the long-lifetime component is quite small, and this component is neglected in the present study. The preexponential factors of the shorter lifetime components are strongly temperature dependent. Both *cis*- and *trans*-PnA show an inversion of the fractional contribution of these two components in a narrow temperature range. The inversion temperature is lower for *trans*-PnA than for *cis*-PnA, both in ethanol and in dodecane. The different temperature behavior of the decay of the two PnA isomers can influence their behavior in phospholipids. In L-α-dimyristoylphosphatidylcholine (DMPC) and L-α-dipalmitoylphosphatidylcholine (DPPC) membranes, the emission is more complex, and more than three components are necessary to describe the fluorescence decay accurately. On the basis of their different behavior in pure solvents, we anticipate that *cis*-PnA will detect the phospholipid transition in DMPC and DPPC at lower temperature. In fact, this effect has been observed, but it has been attributed to different partitioning of the two isomers in the phospholipid phases rather than to their different photophysics as we suggest here.

During the past decade, fluorescence methodologies have been among the most widely utilized approaches to characterizing the physical state of membranes. In particular, fluorescent probes which partition preferentially into membranes have been extensively utilized to monitor lipid fluidity (Shinitzky & Barenholz, 1974).

The naturally fluorescent fatty acids *cis,trans,trans,cis*-9,11,13,15-octadecatetraenoic acid (*cis*-PnA)[1] and *trans*-PnA seemed particularly appropriate for such studies because of their high quantum yields in membranes (relative to the aqueous environment) and their structural analogies to intrinsic membrane components which reduce uncertainties about thier position in the bilayer and render them less perturbing than other more bulky probes.

A different partition coefficient between the gel and liquid-crystal phases of the bilayer has been reported for the two isomers of PnA; these results were based on studies of the decay rates and polarization values of PnA fluorescence in pure solvents (Sklar et al., 1977a) and in synthetic phospholipid membranes (Sklar et al., 1975, 1977b). Specifically, *trans*-PnA is reported to partition preferentially into the gel phase while *cis*-PnA reportedly does not favor one phase over the other. Moreover, in pure solid phospholipids, both PnA isomers are reported to be characterized by a double exponential decay, with a low concentration of short-lived fluorophores, while in pure fluid phospholipids the probes are reported to give a double exponential decay but with a low concentration of long-lived fluorophores. These results have been explained by the persistence of some degree of disorder in the gel phase and some degree of order in the liquid-crystal phase, which are detectable by the PnA isomers. Specifically, the short-lived and long-lived components correspond to fluorophores present in fluid and solid phases, respectively. The prerequisite for such an interpretation is the monoexponential decay of the PnA isomers in pure solvents. Such results have been recently reported in a pulsed lifetime study utiziling synchrotron radiation (Wolber & Hudson, 1981).

The aim of the present study was to verify the decay modes for PnA isomers in pure isotropic solvents and in synthetic phospholipid bilayers to critically evaluate the hypothesis that the different lifetime components can arise from different phases coexisting in model and natural membranes. For our studies, we utilized the powerful new fluorescence methodology of multifrequency cross-correlation phase and modulation fluorometry (Jameson et al., 1984). Using the instrumentation described by Gratton & Limkeman (1983) which allows facile selection of arbitrary light modulation frequencies, we were able to obtained lifetime results with resolutions of several picoseconds. Such multifrequency results permit the resolution of heterogeneous emission into the component lifetimes and the relative fractional weights.

Our results demonstrate that the decay of both PnA isomers in the three pure isotropic solvents utilized is best characterized by three exponentials with relative amplitudes which are highly temperature dependent. These results render questionable the previous partition coefficient determinations for the two PnA isomers in phospholipid bilayers (Sklar et al., 1977b). In addition, the results for the two PnA's in pure phospholipids were more complex than triple exponential decays.

Materials and Methods

cis-PnA and *trans*-PnA were purchased from Molecular Probes Co. The contents of the vials were solubilized in pure ethanol with 0.1% BHT as antioxidant. Prior to use, the PnA–ethanol solution was passed through a silica cartridge (Sep-Pak, Waters Associates, Inc.) and washed once with ethanol in the presence of 0.1% BHT. With this treatment, contaminants and degradation products are reported to remain

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[1] Abbreviations: *cis*-PnA, *cis,trans,trans,cis*-9,11,13,15-octadecatetraenoic acid; *trans*-PnA, *all-trans*-9,11,13,15-octadecatetraenoic acid; BHT, butylated hydroxytoluene; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; DPH, 1,6-diphenyl-1,3,5-hexatriene.

0006-2960/84/0423-5660$01.50/0

absorbed to the silica (Fraley et al., 1978). All operations were carried out in ice under illumination by red light. The probe concentration after the column elution was determined spectrophotometrically in ethanol by using extinction coefficients of $\epsilon_{304.2} = 78\,000\ M^{-1}\ cm^{-1}$ for *cis*-PnA and of $\epsilon_{299.4} = 89\,000\ M^{-1}\ cm^{-1}$ for *trans*-PnA.

Results obtained with this purification method were identical with those obtained with PnA purified on a silver-coated HPLC column and used immediately (Powell, 1982). DMPC and DPPC, from Sigma Chemical Co., were tested for background fluorescence. In all experiments reported here, the background fluorescence was less than 0.5% of the total PnA fluorescence. All solvents were spectroscopic grade and deoxygenated prior to use by bubbling N_2 through the solution for 0.5 h.

Multilamellar phospholipid vesicles were prepared by evaporating aliquots of the phospholipid solutions in chloroform together with the proper amount of the purified PnA–ethanol solution, in the dark and under N_2. Dried samples were resuspended in Dulbecco's PBS previously deoxygenated, capped under N_2, heated above the transition temperature of the phospholipids for 15–30 min, then vortexed for 1 min, and finally capped under N_2 in the cuvette. Final concentrations were 0.5 mM and 3 μM for the phospholipids and the probes, respectively.

Fluorescence measurements were performed with the multifrequency phase and modulation fluorometer described elsewhere (Gratton & Limkeman, 1983). The instrument operates by using the cross-correlation principle introduced by Spencer & Weber (1969). The light source is an argon ion laser whose intensity is modulated sinusoidally with a Pockels cell. The modulation frequencies can be varied continuously from 1 to 160 MHz. Generally, a set of 8–12 modulation frequencies is used in the range most appropriate for the sample under investigation. For each frequency, the phase and the modulation of the fluorescence are measured with respect to a scatter solution (glycogen). Data are collected by an Apple II computer using an ISS01 interface (ISS Inc.). The set of phase and modulation data was analyzed by a nonlinear least-squares routine using software for the Apple II from ISS Inc. The method has been described by Jameson & Gratton (1983) and by Lakowicz et al. (1984). Data are fitted to a sum of exponential terms, each characterized by a lifetime τ and a fractional intensity f. Preexponential factors can be obtained from the fractional intensities by using the relation $\alpha_i = (f_i/\tau_i)/(\Sigma_j f_j/\tau_j)$. The reduced χ^2 parameter is used to judge the "goodness" of the fit. Lakowicz et al. (1984) have discussed the criterion for accepting a decay scheme based on the value of χ^2. Generally, a value less than 3 is considered acceptable while a value on the order of 10 indicates large deviations of the experimentally determined values from the calculated ones. The error in the parameters is calculated by using the correlation matrix of errors as discussed by Lakowicz et al. (1984). All lifetime values and preexponential factors obtained in this work are outside of the nonresolvability limits as calculated by Lakowicz et al. (1984). We can then attribute real significance to the results of the least-squares analysis. Of course, this analysis does not imply that the parameters obtained have physical significance but only that the model used is compatible with the data. In this work, excitation from the argon ion laser was at 351 nm. Emission was observed through a Corning sharp-cut 3-74 filter. Scattered light was observed from a glycogen solution after it passed through an interference filter (PTR Optics) at 351 nm. For the experiments in ethanol at low temperature, a cryostat (Beckman

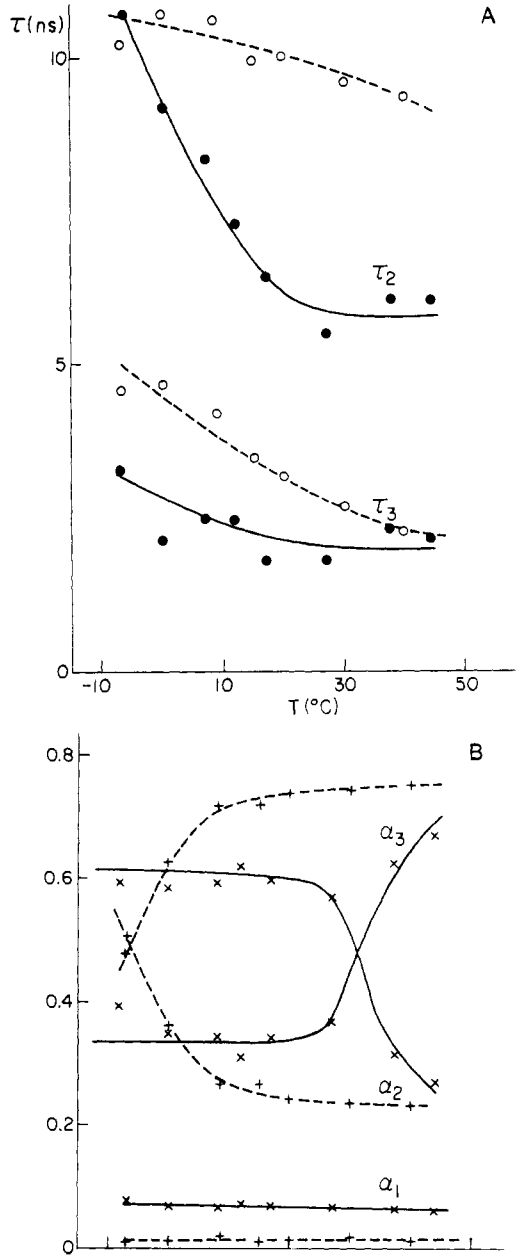

FIGURE 1: Lifetime values (τ) (A) and associated preexponential factors (α) (B) vs. temperature of *cis*-PnA (● and ×) and of *trans*-PnA (○ and +) in dodecane, as determined by a three-component analysis.

196778), modified for fluorescence experiments, was used. In this case, the internal scatter of the sample was used as reference, viewed by using the interference filter at 351 nm. Fluorescence spectra were recorded by using the photon-counting microcomputer-controlled fluorometer described by Gratton & Limkeman (1983a).

Results

Phase and modulation lifetimes for *cis*- and *trans*-PnA in dodecane were determined at 11 modulation frequencies in the range of 1–80 MHz and at various temperatures. These data were analyzed by the nonlinear least-squares routine described under Materials and Methods. The best fits, with low χ^2 values, were obtained only by using a three-component analysis. The three liftime values were approximately 40, 9, and 2 ns for *cis*-PnA and 50, 10, and 3 ns for *trans*-PnA (Figure 1) and were temperature dependent. The longest lifetime value was not reported in the plots. The longest lifetime component had very low, temperature-independent

Table I: Three-Component Analysis of *cis*-PnA in Cyclohexanol at 25 °C[a]

cis-PnA									
τ_1 (ns)	f_1	α_1	τ_2 (ns)	f_2	α_2	τ_3 (ns)	f_3	α_3	χ^2
2.14	1.0	1.0							34.90
355.49	0.046	0.0002	2.1	0.964	0.999				6.72
6067.58	0.028	1×10^{-5}	2.4	0.815	0.667	0.98	0.157	0.498	0.67

[a] Lifetimes, τ; fractional intensities, f; preexponential factors, α.

preexponential factors of 0.06 and 0.01 for *cis*- and *trans*-PnA, respectively. The intermediate lifetime value is associated with a temperature-dependent preexponential factor and is the main component at low temperatures. The shortest lifetime component is also associated with a temperature-dependent preexponential factor and gives the major contribution to the emission at high temperatures. As a consequence, the plots (Figure 1) of the PnA's preexponential factors associated with the two main lifetimes show a crossing point. This point occurs at different temperatures for *cis*- and *trans*-PnA in dodecane, 32 and 7 °C, respectively.

Lifetime values and the associated preexponential factors obtained for *cis*- and *trans*-PnA in ethanol are reported in Figure 2. Three temperature-dependent lifetime components are determined in this case also. The associated preexponential factors show a behavior similar to that reported in dodecane. The longest lifetime preexponential factor is very small (around 0.005 for both PnA isomers) and constant with temperature. The preexponential factors associated with the intermediate lifetime value (τ_2) and with the shortest lifetime value (τ_3), α_2 and α_3, respectively, are temperature dependent. In ethanol, as in the case of PnA isomers in dodecane, a crossing point for α_2 and α_3 is present and occurs at different temperatures for *cis*- and *trans*-PnA, 8 and −35 °C, respectively.

From the dodecane and ethanol experiments, it is evident that at high temperatures the shortest lifetime component is predominant, while at low temperatures the intermediate lifetime component is associated with the highest preexponential factor. The α_2 and α_3 values inversion occurs in a narrow temperature range. Such an inversion temperature depends on the solvent, and in the same solvent occurs at a lower temperature for *trans*-PnA and at a higher temperature for *cis*-PnA.

Results obtained for *cis*-PnA in cyclohexanol at 11 modulation frequencies and at 25 °C are reported in Table I. The decrease in the χ^2 value upon going from a one-component to a three-component analysis is evident. This result is not affected by the probe concentration or by the emission wavelength variation.

Results of a three-component analysis of the lifetime data for *cis*- and *trans*-PnA in DMPC bilayers are reported in Figure 3 as a function of temperature. One also observes that with a three-component analysis the χ^2 value remains fairly high when compared to those for the pure isotropic solvents, especially in the case of *trans*-PnA. Nevertheless, we may conclude that the inversion of the α_2 and α_3 values occurs for *cis*-PnA in DMPC but not with *trans*-PnA in DMPC; these results are consistent with the observation of a lower temperature crossing point for *trans*-PnA in pure solvents. At the DMPC transition temperature with *trans*-PnA, we can only observe an increase in the shortest lifetime preexponential factor. With *cis*-PnA, the inversion of the α_2 and α_3 values also occurs a few degrees centigrade before the known T_c of DMPC (23 °C), followed by a pronounced increase of the α_3 value.

The shortest lifetime component for both PnA isomers is almost temperature independent and does not sense the

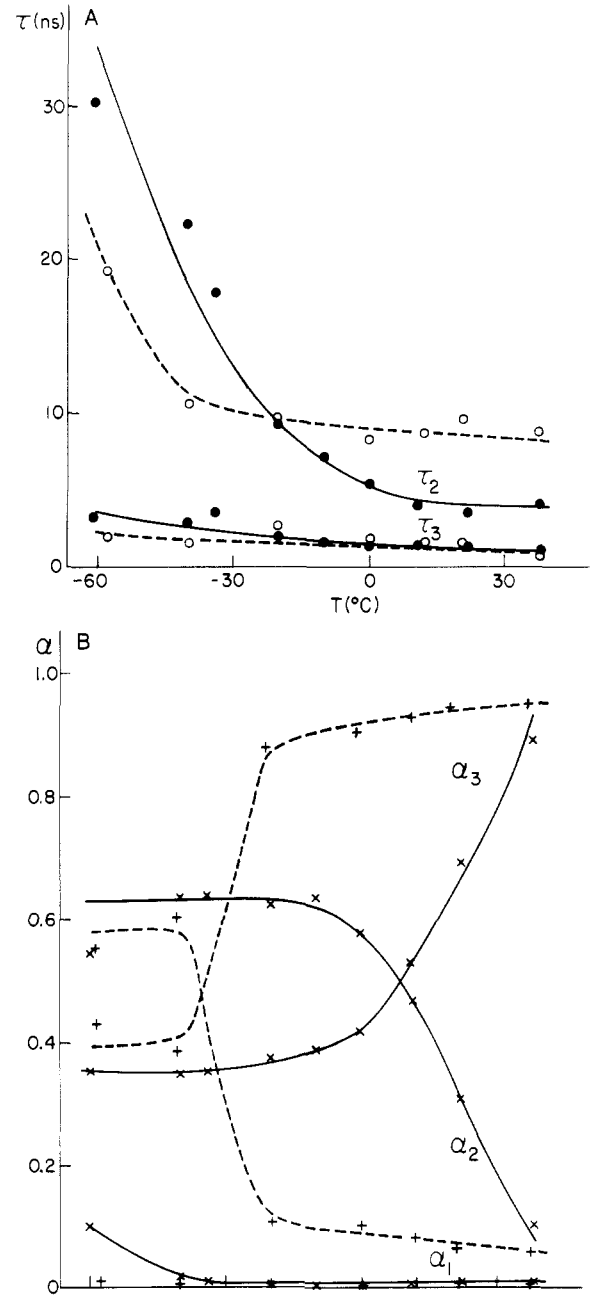

FIGURE 2: Lifetime values (τ) (A) and associated preexponential factors (α) (B) vs. temperature of *cis*-PnA (● and ×) and of *trans*-PnA (○ and +) in ethanol, as determined by a three-component analysis.

phospholipid transition. The same situation holds for the longest lifetime component which is not reported in the plots because of the very low associated preexponential factor. The intermediate lifetime component can detect the phospholipid transition with both *cis*- and *trans*-PnA, as evidenced by the 2-fold change in magnitude at the T_c. Almost the same behavior was found for the two probes in DPPC vesicles, and all the considerations given above are also valid in this case, as can be observed from Figure 4.

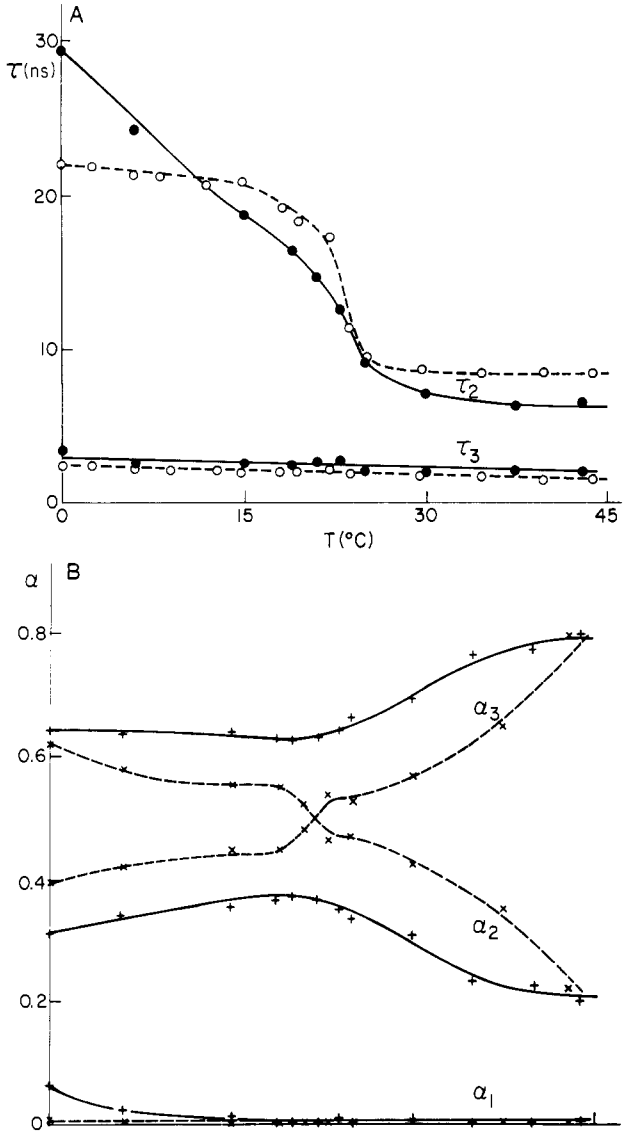

FIGURE 3: Lifetime values (τ) (A) and associated preexponential factors (α) (B) vs. temperature of *cis*-PnA (● and ×) and of *trans*-PnA (○ and +) in DMPC, as determined by a three-component analysis.

Table II: Comparison between Three- and Four-Component Analysis of *trans*-PnA in DPPC[a]

temp (°C)	component	τ (ns)	f	α	χ^2
35.5	1	496.9	0.188	0.005	
	2	22.6	0.703	0.413	
	3	2.5	0.108	0.582	16.2
	1	980.0	0.151	0.002	
	2	33.0	0.472	0.168	
	3	13.0	0.306	0.277	
	4	1.5	0.071	0.554	0.9
48	1	994.5	0.288	0.001	
	2	11.3	0.356	0.161	
	3	2.2	0.356	0.838	10.5
	1	1347.4	0.271	0.001	
	2	33.0	0.081	0.012	
	3	8.6	0.332	0.193	
	4	2.0	0.316	0.793	5.7

[a] Lifetimes, τ; fractional intensities, f; preexponential factors, α.

A four-component analysis has been attempted for the *trans*-PnA in DPPC data at 35.5 °C. The results show the decrease of χ^2 to a very good value (Table II) and the splitting of the intermediate lifetime value of 22.6 ns into two components of 33.0 and 13.0 ns, respectively. The four-component analysis for the same sample but at 48 °C, above the DPPC

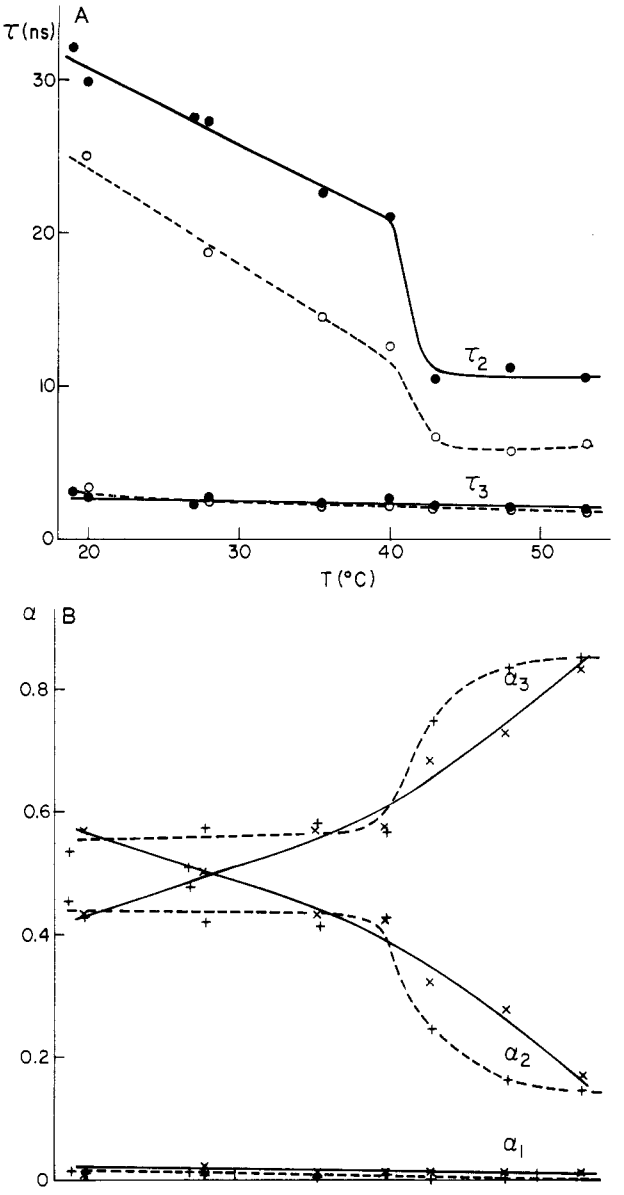

FIGURE 4: Lifetime values (τ) (A) and associated preexponential factors (α) (B) vs. temperature of *cis*-PnA (● and ×) and of *trans*-PnA (○ and +) in DPPC, as determined by a three-component analysis.

transition, shows the decrease of the χ^2 value and the splitting of the intermediate lifetime component in two, with new values of 33.0 and 8.6 ns. The DPPC transition seems to involve the third lifetime component with its value decreasing from 13.0 to 8.6 ns, but the associated preexponential factor's redistribution is more relevant. Above the transition temperature, the fourth lifetime component shows the maximum concentration, the preexponential factor of the third component decreases, and the second component almost disappears.

In all our determinations of *cis*- and *trans*-PnA lifetimes, in solvents and in phospholipid bilayers, we did not attribute any relevance to the longest lifetime value because of its negligible concentration and because of the difficulty inherent in the exact determination of such a long-lifetime value with the frequency set utilized in our experiments.

Discussion

The coexistence of different phospholipid phases in plasma membranes has been reported (Klausner et al., 1980a,b; Pessin & Glaser, 1980; Dratz, 1979). These observations have led to the hypothesis that the segregation of phospholipids into domains in membranes is relevant to the control of physio-

logical membrane functions and may be implicated in abnormal cell growth.

The development of fluorescence methodologies able to distinguish and quantitate the segregation of lipids into domains is thus highly desirable. This study, however, suggests that parinaric acid probes may not be suitable for such purposes, due to the intrinsic complexities of their emission. In particular, the observed three-component decay for both PnA isomers in pure, isotropic solvents hinders interpretation of the more complex decays observed in model membrane systems. Moreover, the relative preexponential factors of these components vary with temperature and solvent composition. These variations combine to produce changes in the observed steady-state polarization values in accordance with Weber's rules for the additivity of anisotropies (Weber, 1952).

Fluorescence polarization from *cis*-PnA has been reported to detect the DPPC and DMPC transitions about 1 °C lower than does *trans*-PnA (Sklar et al., 1977a). This differential sensitivity can be explained with reference to the time-resolved results shown in Figures 3 and 4. Specifically, in both phospholipid systems, *cis*-PnA demonstrates the inversion of the α_2 and α_3 values just a few degrees centigrade before the transition with the preexponential factor relative to the shortest lifetime value becoming predominant. *trans*-PnA does not exhibit such an inversion; hence, the average lifetime of *cis*-PnA will decrease at a lower temperature than does *trans*-PnA.

The study of the PnA's emission in pure isotropic solvents indicates the potential of multifrequency phase and modulation fluorometry in analyzing a heterogeneous emitting system. Monoexponential decays for *cis*- and *trans*-PnA were obtained by using the time-correlated single photon counting technique coupled with synchrotron radiation (Wolber & Hudson, 1981), but even with the excellent temporal properties of that light source, the complex heterogeneity of these probes was not observed.

In the present study, we do not attempt to explain the origin of the three lifetimes of PnA isomers and their values and the reasons for the behavior of the associated preexponential factors. One may speculate, however, that the presence of three lifetimes can be due either to different molecules or to different excited states of the PnA molecules, the population of which is determined by temperature-dependent barriers. The absolute values of the lifetimes are probably related to the polarity or polarizability of the solvents, which is known to affect the lifetime value of conjugated polyenes (Hudson & Kohler, 1974). Instead, there is no apparent relation between the viscosity of the solvent and the lifetime values. This observation excludes that dynamical conformational effects in the ground or in the excited state can be responsible for the heterogeneity of the emission. Similar conclusions are also reported for DPH (Zannoni et al., 1983). Formation of dimers or larger aggregates is also excluded since our results were independent from PnA's concentration.

The interpretation of the lifetime data of PnA obtained in DMPC and DPPC vesicles is, at the moment, more difficult, since the PnA's lifetime decays fit a triple exponential in pure solvents, and in phospholipid vesicles an even more complex decay is expected. A four-component analysis of PnA isomers in DMPC and DPPC gives a better fit than that obtained with three components, but we do not attribute much physical significance to the values of the components obtained from a four-component fit. We believe that a much greater accuracy in the lifetime determinations is needed in order to decompose a decay into more than three components.

In conclusion, we believe that the complex decays displayed by PnA isomers render questionable previous speculations on the property of these isomers to partition differentially in phospholipid phases. This result does not imply that lifetime values of the PnA probes are not sensitive to phase transitions in phospholipids. On the contrary, the average lifetime of PnA isomers, as well as the polarization values, dramatically changes during the phase transition. However, we believe that the difference between the two PnA isomers can be better explained on the basis of different photophysics rather than invoking differential partitioning.

Acknowledgments

We are deeply indebted to Dr. D. M. Jameson and Dr. R. P. Holmes for many useful comments and critical reading of the manuscript. We thank Drs. R. P. Holmes and T. Smith for providing us with HPLC-purified PnA.

Registry No. *cis*-PnA, 593-38-4; *trans*-PnA, 18841-21-9; DMPC, 18194-24-6; DPPC, 63-89-8.

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