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Conjugated Polyene Fatty Acids on Fluorescent Probes: Spectroscopic Characterization†

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ABSTRACT: This paper is the first in a series which extends introductory studies of parinaric acid and its phospholipid derivatives as membrane probes (Sklar, L. A., Hudson, B., and Simoni, R. D. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1649; (1976), *J. Supramol. Struct. 4,* 449). Parinaric acid has a conjugated tetraene chromophore and exhibits many spectroscopic properties common to linear polyenes. Its absorption spectrum is characterized by a strong near-ultraviolet transition with vibronic structure, which is strongly affected by solvent polarizability. The fluorescence emission occurs at considerably lower energy than the absorption and the wavelength of the emission is nearly independent of the solvent. The fluorescence quantum yield and lifetime are strongly affected by temperature and solvent. These spectral features are interpreted in terms of an excited electronic-state order such that a weak transition occurs at longer wavelengths than the strongly allowed transition which dominates the absorption. The sensitivity of the fluorescence quantum yield and lifetime to environment is shown to be due primarily to variations in the nonradiative rate, although changes in the radiative rate constant are also observed and interpreted. The absorption spectrum ($\epsilon_{max} > 65\,000$) is in the 300–320-nm range, a region relatively free of absorption due to intrinsic biological chromophores. Shifts of several nanometers are characteristic of different environments. These shifts are compared to similar effects observed for a series of diphenylpolyenes for which new data are given and are correlated using a simple but adequate theory of solvent shifts. The intrinsic (or radiative) fluorescence lifetime is near 100 ns in a wide variety of environments. This is much longer than the intrinsic lifetime calculated from the absorption spectrum and strongly supports the proposed excited-state order.

Probe techniques have provided much useful information about structure–function relationships in membranes (Radda, 1975; Waggoner, 1976; McConnell, 1976; Griffith and Jost, 1976). Although these probe techniques are undoubtedly sensitive to subtle environmental changes, considerable uncertainty exists concerning the perturbing influence that is exerted by the introduction of bulky substituents into a membrane. The location, orientation, and specificity of probe interactions with the membrane components are often difficult to ascertain. In this series of reports, we wish to define the physical properties and establish the utility of a new class of fluorescent probe molecules—the linear polyene fatty acids.

This paper examines the spectroscopy of two isomers of parinaric (octadecatetraenoic) acid, α(9,11,13,15-*cis,trans,trans,cis*) and β(9,11,13,15-*all-trans*). Particular reference is made to the relationship between parinaric acid spectroscopy and the body of knowledge available for linear polyenes in general (Hudson and Kohler, 1974). The diphenylpolyenes serve as reference compounds in this regard and new data are presented on the effect of solvent polarizability on the absorption spectra of these compounds. The two following papers in this issue apply these results to studies of model lipid systems and *Escherichia coli* membranes.

Other linear polyenes have been used as probes of membrane structure, including diphenylhexatriene (Shinitzky and Barenholz, 1974; Andrich and Vanderkooi, 1976), the macrolide polyene antibiotics filipin and amphotericin (Bittman et al., 1974), and various retinal and carotenoid molecules (Chance and Baltscheffsky, 1975; Radda and Smith, 1970). Although polyene spectroscopy has been an area of investigation for 40 years (Hausser et al., 1935), a complete spectroscopic characterization has been accomplished for very few polyene molecules. A recent review considers several of the experimental and theoretical inconsistencies that have been dormant in this field (Hudson and Kohler, 1974). It is appropriate here to mention that approximate quantum mechanical (molecular

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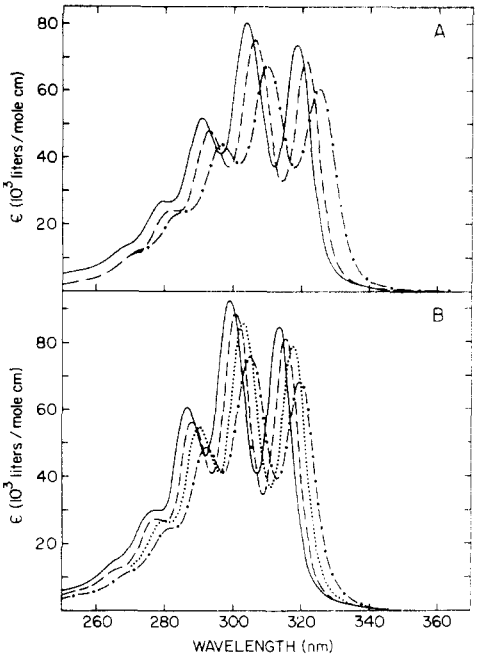

FIGURE 1: Absorption spectra of *cis*- (A) and *trans*-PnA (B) in various solvents. The concentration is $1.00 \pm 0.01 \times 10^{-5}$ M and the temperature is 25 °C in all cases. In A (*cis*-PnA), the solvents are methanol (—), cyclohexane (- - -), and chloroform (- • -). In B (*trans*-PnA), the solvents are methanol (—), decane (- - -), *p*-dioxane (· · ·), and chloroform (- • -). Absorption spectra were recorded on a Cary 14 spectrophotometer.

orbital) theories predict that the ^1B_u state responsible for the strong polyene absorption is the lowest lying singlet state, but there is considerable evidence (Hudson and Kohler, 1972, 1973, 1974; Christensen and Kohler, 1975) that the lowest excited singlet state for many polyenes is a state with ^1A_g symmetry (Schulten and Karplus, 1972).

This work presents the results of a spectroscopic characterization of both isomers of parinaric acid. Solvent and temperature dependence of the absorption spectra, fluorescence lifetime, and quantum yield have been studied. Our results are consistent with the electronic state ordering in which a ^1A_g state is the lowest singlet. A strong transition of oscillator strength ~1.5 has absorption maxima in the 300-nm region, but the intrinsic fluorescence lifetime reported here is 100 ns, much longer than estimated on the basis of the integrated absorption (Strickler and Berg, 1962). The absorption spectrum is sensitive to solvent and temperature in a fashion similar to that observed for a variety of other linear polyenes. The changes in the fluorescence quantum yield and lifetime are due largely to changes in the nonradiative decay rate for the excited state.

Materials and Methods

Seeds of the plant *Parinarium glaberrima* were obtained with the kind assistance of D. Koroveibau of the Department of Agriculture, Suva, Fiji, and R. H. Philips of the Nasese Nursery, Suva, Fiji. Parinaric acid (PnA[1]) was prepared from the seed oils as described in the following paper of this issue. Freshly recrystallized polyene fatty acids were dried under argon, weighed rapidly, and dissolved in absolute ethanol (1 mg/mL of *cis*-PnA and 0.5 mg/mL of *trans*-PnA in the presence of 1 mg/100 mL of BHT). Crystals dissolved completely only when fresh and when exposure to light and oxygen was limited; otherwise, significant amounts of polymeric material appeared. Solutions stored at −20 °C in darkness have been relatively stable (optical density changes 5–10%) for periods of months. No significant amount of isomerization has been observed under these conditions of storage. Commercial samples of the diphenylpolyenes with one to four double bonds were used. Diphenyldecapentaene was synthesized according to Kuhn and Winterstein, 1928. The compounds with six and eight double bonds were a gift from Prof. William Moomaw of Williams College. The problem of impurities in these materials has been discussed elsewhere (Hudson and Kohler, 1973).

Fluorescence emission spectra were compared to 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (Me_2POPOP) in cyclohexane (Q = 0.93) for quantum-yield measurements (Berlman, 1971). The solutions used had an optical density of approximately 0.1 at the excitation wavelength. Similar solutions, containing equimolar BHT, in various solvents, were used in the measurements of fluorescence intensity vs. temperature. For these measurements, an X-Y plotter was used, with a thermocouple to drive the x axis, and the fluorimeter output scaled through an electrometer to drive the y axis. The absorption spectrum of BHT is insignificant at the excitation wavelength of PnA (314–320 for trans, 319–325 for cis) and the emission spectrum of BHT was insignificant at the emission wavelength of 410–420 nm for PnA. Corrected fluorescence emission spectra and quantum yields measured against anilinonaphthalenesulfonate (ANS) as standard (Q = 0.37 in absolute ethanol) (Stryer, 1965) were determined at Yale University in the laboratory of Dr. Lubert Stryer. Lifetimes were measured by pulse-counting techniques as previously described (Yguerabide, 1972). For these measurements, the solutions had an optical density of approximately 0.1 at the excitation peak, and no additional BHT was used. Samples were bubbled with argon and, when necessary, the temperature was regulated with a circulating water bath. Temperatures were monitored in reference cuvettes, and all samples and references were allowed to equilibrate in the water bath. No significant sample decomposition was noted. An excitation filter (Corning 7-54, UV transmission) and emission filter (Corning 4-76, clear glass) were used. Spectroscopic grade solvents were used where available, and emission blanks with identical conditions of temperature and preparation were recorded.

Results and Discussion

Absorption Spectroscopy. The absorption spectra of *cis*- and *trans*-PnA in several solvents are shown in Figure 1. As the solvent polarizability increases, a red shift in the spectrum is observed concordantly with a decrease in the extinction coefficient. The peak positions and maximum extinction coefficients for both geometric isomers are given in Table I for selected solvents. A more extensive listing of data of this type is given in the Supplementary Material. The solvents are listed in order of increasing α, defined as $(n^2 - 1)/(n^2 + 2)$, where n is the refractive index. This quantity is related to the solvent polarizability.

The correlation of peak position with solvent polarizability is common to all linear polyenes. Figure 2 shows the solvent dependence of the first absorption peak position for several linear polyenes. The intercepts at $\alpha = 0$ correspond to gas-phase excitation energies. The values obtained for the diphenylpolyenes are discussed elsewhere (Hudson et al., 1976; Yip et al., 1976). The upper section of Figure 2A shows data

[1] Abbreviations used are: PnA, parinaric acid; BHT, di-*tert*-butyl-4-methylphenol; ANS, 8-anilino-1-naphthalenesulfonate; Me_2-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene; DMF, dimethylformamide; UV, ultraviolet.

TABLE I: Absorption Spectral Data for *cis*- and *trans*-Parinaric Acid.

Solvent	λ_1 (nm)	λ_2 (nm)	$\epsilon(\lambda_2)$ (L mol^{-1} cm^{-1})
		cis-Parinaric Acid	
Methanol	318.6	303.8	79 000
Ether	318.8	304.0	81 000
Ethanol	319.2	304.2	78 000
Hexane	318.9	304.0	78 000
1-Butanol	320.6	305.6	73 000
Decane	320.8	305.8	74 000
p-Dioxane	322.7	307.6	74 000
Chloroform	324.8	309.8	68 000
Benzene	325.3	310.0	67 000
		trans-Parinaric Acid	
Methanol	313.0	298.6	92 000
Ether	313.5	299.0	93 000
Ethanol	313.8	299.4	89 000
Hexane	313.5	298.9	91 000
1-Butanol	315.2	300.6	85 000
Decane	315.3	300.7	88 000
p-Dioxane	317.3	302.7	85 000
Chloroform	319.6	304.7	76 000
Benzene	320.0	305.2	77 000

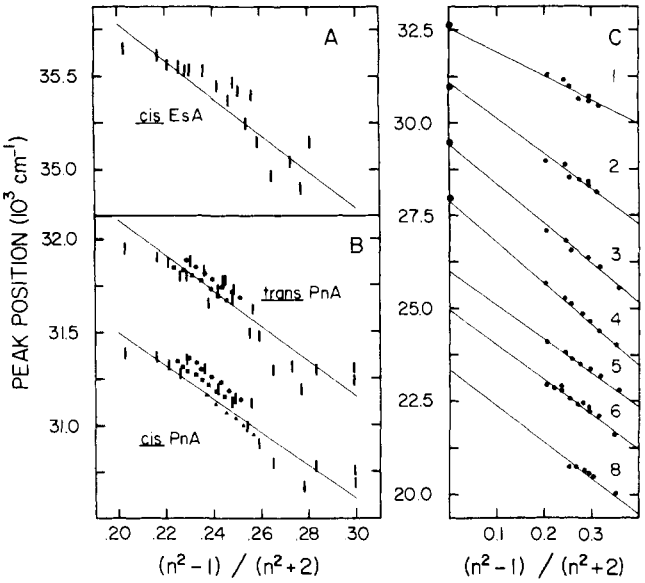

FIGURE 2: Solvent shifts of linear polyene absorption maxima as a function of the solvent polarizability. (A) α-eleostearic acid (*cis*-EsA); (B) *trans*-PnA (upper line) and *cis*-PnA (lower line); (C) the diphenylpolyenes. The numbers at the right in panel C represent the number of polyene double bonds. The longest wavelength band maxima (Table I) are plotted as $1/\lambda_1$ vs. $\alpha = (n^2 - 1)/(n^2 + 2)$. The points at the left axis ($\alpha = 0$) of panel C are measured gas-phase values. The scales for A and B are expanded by a factor of five along both axes compared to those of C. The peak-position measurements are the average of three or more independent determinations. The reproducibility is estimated to be 0.2 nm or about 20 cm^{-1}. In B, data are shown both for various solvents and for particular solvents whose polarizability is varied by changing the temperature. The solvents are *n*-decane (circles), 1-butanol (squares), and *p*-dioxane (triangles). The temperatures used were in the range from 4 to 90 °C. The solvent refractive index was calculated from the Lorentz–Lorenz equation using the value at 20 °C and the density as a function of temperature (Calingaert et al., 1941; Lange, 1961). For *p*-dioxane, the refractive index was measured as a function of temperature.

for α-eleostearic acid (*cis,trans,trans*-9,11,13-octadecatrienoic acid), while the bottom section shows data for *cis*- and *trans*-PnA. Figure 2B shows data for the series of diphenylpolyenes with one (at top of figure) through six and eight (at bottom) double bonds.

Figure 2B contains data for variations in α due to thermal expansion for several solvents in addition to the points obtained by changing the solvent. The slope of these lines is approximately the same for both types of data and is 10 000 ± 1000 cm^{-1} for all polyenes. A table of the least-square slopes and intercepts for all of the lines of Figure 2 is given in the Supplementary Material.

There is a strong linear correlation between the absorption peak position and the maximum extinction coefficient for both *cis*- and *trans*-PnA. This is seen in the spectra of Figure 1 and the data of Table I. A plot of this correlation is given in the Supplementary Material. The points obtained for *cis*-PnA bound to bovine serum albumin and incorporated into phospholipid bilayers also fit this correlation. The extinction coefficient is anomalously low for carbon tetrachloride solutions, probably due to aggregation.

Interpretation of Absorption Spectra. The shift of the absorption spectra of linear polyenes in response to solvent and temperature variation can be explained in terms of simple theories of solvent shifts of electronic spectra (Basu, 1964; Amos and Burrows, 1973). Application of these simple theories to linear polyene spectra has shown that the strong absorption transition of these nonpolar chromophores shifts primarily in response to the polarizability of the environment and little or not at all in response to the solvent polarity (Hudson and Kohler, 1973). This situation arises in part because neither the ground nor the excited states of these linear polyenes have a dipole moment and because the polyene electronic transition dipole is very large. The polarizability of the solvent is a measure of the response of the solvent electron clouds to the high-frequency transient dipole induced by an electronic transition.

For a nonpolar molecule in a nonpolar solvent, the spectral shift is determined by the difference in the dispersion energy for the ground and excited state. This has the approximate form

$$\bar{\nu} = \bar{\nu}^0 - k\alpha \qquad (1)$$

where $\bar{\nu} = 1/\lambda$, $hc\bar{\nu}^0$ is the gas phase ($\alpha = 0$) excitation energy, and α has been defined above. The constant k is large for strongly allowed transitions.

TABLE II: Summary of Fluorescence Data for *cis*- and *trans*-Parinaric Acid.

Solvent	*cis*-Parinaric Acid					*trans*-Parinaric Acid		
	τ (ns)	Q^a	$\theta(K^{-1} \times 10^3)^b$	$k_c(10^7/s)$	$k_{nr}(10^7/s)$	τ (ns)	Q^a	$\theta(K^{-1} \times 10^3)^b$
Cyclohexane	3.7	0.044		1.2	26	(3)[c]	0.031	
Hexane	4.5	0.046	0.29	1.0	21		0.027	
Octane	5.1							
Decane	5.2	0.054	0.23	1.0	18	3.1	0.031	0.28
Tetradecane	5.0					3.2		
Methanol	1.3	0.017	0.26	1.3	76	<1	0.009	0.38
Ethanol	1.9	0.023		1.2	52	<1	0.012	0.35
Propanol	2.1	0.020		1.0	47	<1	0.016	
Butanol	2.5	0.025	0.21	1.0	39	1.5	0.017	0.25
p-Dioxane	3.2	0.051	0.25	1.6	29	(2.5)[c]	0.025	0.30
Ether	5.1	0.052		1.0	19	(4)[c]	0.028	
Dimethyl formamide	1.8	0.030		1.7	53		0.010	
Chloroform	1.5	0.020	0.26	1.3	66		0.010	

[a] Fluorescence quantum yield relative to Me_2POPOP ($Q = 0.93$). Values obtained relative to ANS ($Q = 0.37$) for *cis*-parinaric acid were 0.045 (decane), 0.020 (ethanol), and 0.047 (*p*-dioxane), and for *trans*-parinaric acid were 0.029 (decane) and 0.011 (ethanol). The values are lower than the Me_2POPOP values by a factor of 1.12 ± 0.06. [b] Additional θ values for *cis*-parinaric acid were $0.25 \times 10^{-3}\ K^{-1}$ (amyl alcohol) and for *trans*-parinaric acid were $0.27 \times 10^{-3}\ K^{-1}$ (heptane) and $0.28 \times 10^{-3}\ K^{-1}$ (amyl alcohol). [c] Lifetimes in parentheses are approximate (± 0.5 ns).

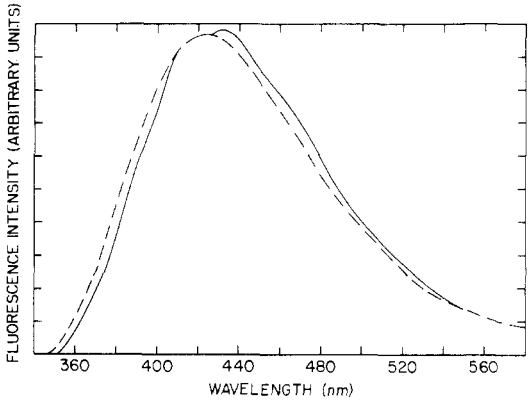

FIGURE 3: The fluorescence emission spectrum of *cis*- (—) and *trans*-PnA (- - -) in decane at 25 °C. Solutions with less than 0.1 OD at the excitation peak were deoxygenated with nitrogen. Excitation was at the long-wavelength absorption maximum (319 nm cis and 314 nm trans) with slit width 2 nm, and emission (with slit width 2 nm) was scanned. The corrected spectra are plotted in arbitrary fluorescence units vs. wavelength.

A large number of assumptions are involved in the derivation of eq 1. For polar solvents, a term involving the solvent dipole moment and the solute polarizability change on excitation must, in principle, be included. Deviations from eq 1 are therefore anticipated. The scatter in the data of Figure 2 is clearly not random, is much larger than the experimental error, and represents contributions to the solvent shift from nondispersive interactions.

The reasons for systematic variation of the extinction coefficient with solvent shift are not well understood. The trend of the data (low ϵ for high n) is opposite to that expected on the basis of an older theory (Chako, 1934) but may be consistent with more recent treatments (Schuyer, 1953).

Fluorescence Spectroscopy. Figure 3 shows the corrected emission spectra of both isomers of PnA in decane at 25 °C. The emission origin is at about 350 nm and a broad maximum (at about 422 nm for trans and 432 nm for cis) with little fine structure is observed. The emission spectrum is essentially independent of the particular solvent.

The fluorescence quantum yield and lifetime of PnA were determined in several solvents, as shown in Table II. Two methods for evaluating the fluorescence quantum yield were used, as described under Materials and Methods. The more reliable technique, which has an estimated accuracy of 10%, is the comparison of PnA fluorescence intensity to ANS, in ethanol, a standard of $Q = 0.37$, on the corrected fluorimeter of Dr. Lubert Stryer at Yale University. In our own laboratory, using Me_2POPOP in cyclohexane, because of similar emission spectrum distribution to PnA, we have compiled a more complete list of quantum yields, which are systematically 10–20% larger than those determined by comparison to ANS.

Based on the Me_2POPOP data, we have calculated the emissive and the nonradiative rate constants for *cis*-PnA, as well as the intrinsic lifetime of the fluorescence. It is seen that, with the exception of two solvents, dioxane and dimethylformamide, the radiative rate constant is nearly constant at $10^7\ s^{-1}$, while the fluorescence lifetime and nonradiative rate constant vary by a factor of four. The data for *trans*-PnA, although less complete, is similar. For *trans*-PnA in dipalmitoylphosphatidylcholine vesicles at 20 °C, $Q = 0.3$ and $\tau = 30$ ns. These are the largest values yet obtained. Again, $\tau_0 = 100$ ns.

A plot of log (fluorescence intensity) vs. reciprocal temperature results in a nearly linear relationship (Figure 4). We have defined the parameter θ, as the range in reciprocal degrees (K^{-1}) over which the fluorescence intensity varies by a factor of two. If T_2 is the temperature at which Q is half its value at T_1, then $\theta = (1/T_1) - (1/T_2)$. For $\theta = 0.25 \times 10^{-3}\ K^{-1}$ and $T_1 = 20$ °C (293 K), $T_2 = 43$ °C (316 K). Typical values of θ, as shown in Table II, are in the range $0.2–0.4 \times 10^{-3}\ K^{-1}$. Generally, the values observed for *cis*-PnA are 20% less than those for *trans*-PnA. We have indicated previously (Sklar et al., 1975, 1976) that for these molecules dissolved in phospholipids above the transition temperature the temperature dependence of their fluorescence is very similar to the behavior observed in long-chain hydrocarbon solvents.

Figure 4 also shows the temperature dependence of the fluorescence lifetime of *cis*-PnA in decane. When log τ is plotted vs. $1/T$, a nearly linear relationship results. The characteristic slope, θ_τ, is defined analogously to the temperature dependence of the quantum yield, θ. These slopes, θ and θ_τ, are remarkably similar. The quantum yield Q is related to

the lifetime by the expression:

$$Q = k_e\tau = \tau/\tau_0 \quad (2)$$

where $k_e = 1/\tau_0$ is the intrinsic emissive rate constant. Since Q and τ vary in approximately parallel fashion with temperature, it is obvious that k_e, the emissive rate constant must be largely unaffected by changes in temperature or the associated polarizability variation. This result is consistent with the approximate independence of k_e to the nature of the solvent, as illustrated in Table II. The approximate constancy of k_e indicates that the lifetime of parinaric acid may be estimated from quantum-yield measurements alone. This is extremely useful in fluorescence polarization measurements where the lifetime must be known in order to interpret the results in terms of changes in rotational mobility.

Interpretation of Fluorescence Data. The measurable quantities Q and τ are related to the more fundamental quantities k_e, the emissive rate constant, and k_{nr}, the nonradiative rate constant, by the expression

$$Q = \frac{k_e}{k_e + k_{nr}} = k_e\tau \quad (3)$$

where $k_e = 1/\tau_0$.

The emissive rate constant k_e or, equivalently, the radiative lifetime τ_0, is roughly a constant for a variety of solvents (Table II) and in decane over the temperature range of 12 to 66 °C (Figure 4). A table of the value of Q and τ and the derived quantities k_e and k_{nr} for *cis*-PnA in decane as a function of temperature is included in the Supplementary Material. The extreme values of k_e differ by a factor of 1.7, while k_{nr} varies by a factor of 4.2. With increasing temperature, k_{nr} increases while k_e decreases.

On the other hand, it is clear that the observed variation in k_e (or τ_0) is real. For instance, the value for DMF and dioxane is clearly less than the value for decane or propanol by 40% (Table II) and, in Figure 4, the lines showing the variation in Q and τ are not parallel. This variability in k_e has been previously reported for a variety of polyenes (Hudson and Kohler, 1974; Dalle and Rosenberg, 1970; Birks and Birch, 1975; Radda and Smith, 1970). This is of interest because of its relevance to the use of polyenes as probes of rotational dynamics in biological systems and because it is related to the nature of the excited electronic states of polyenes. The strongly allowed electronic transition of linear polyenes is not the lowest energy transition and the excited state produced by this transition is not the state from which fluorescence originates. Polyenes have a weak transition below the strongly allowed transition whose intensity is primarily borrowed from the allowed transition by vibronic mixing and other molecular distortions. The net result is that the intrinsic fluorescence lifetime for a polyene, τ_0, is much longer than expected on the basis of its integrated (strong) absorption spectrum. Furthermore, τ_0 depends on the solvent polarizability. This is because the intensity borrowed from the strong transition depends on the energy difference between the excited states (as ΔE^{-2}) and this energy difference is a function of solvent polarizability via differential solvent shifts of the form discussed above. We may relate τ_0 to the gap between the excited states approximately as follows (Hudson and Kohler, 1973)

$$\tau_0 = \frac{1}{k_e} = \Gamma(\Delta E)^2/F \quad (4)$$

In this expression, ΔE is the effective energy difference between the excited ^1A_g state, which gives rise to the fluorescence, and the excited ^1B_u state, which gives rise to the strong absorption, and F is the oscillator strength of the strong absorption transition from the ground state to the ^1B_u state. Γ is a collection of constants including the conversion factor between the oscillator strength and the emissive rate constant for the fluorescence transition and the perturbation terms which mix the excited ^1A_g and ^1B_u states. These perturbation terms may be vibronic in origin or due to the fact that PnA does not have a center of symmetry. These contributions to the excited state mixing will not depend significantly on the solvent. On the other hand, the local dipolar field of the solvent may also contribute to this mixing and thus Γ will depend to some extent on the polarity of the molecular environment. Data for various solvents are therefore difficult to interpret and the rather erratic behavior of τ_0 in various solvents (Table II) is expected if not predictable. For a particular nonpolar solvent, however, Γ will be roughly independent of temperature. The observed variation in τ_0 is due to thermal expansion and a resulting decrease in α with increasing temperature. The dominant effect is the variation in ΔE. As the temperature is increased, the ^1B_u state moves to higher energy (Figure 2), while the ^1A_g state remains at a constant energy. As a result, ΔE increases and τ_0 increases with increasing temperature. This dominant effect is partially compensated by small opposing variations in Γ and F. Quantitative application of eq 4 to the data is given elsewhere (Andrews and Hudson, manuscript in preparation). Equation 4 is a crude approximation, since it assumes that all of the intensity of the fluorescence transition is borrowed from a single higher excited state and that this mixing may be described by a single mixing coefficient and energy gap. However, we believe that the major source of variation of k_e with temperature has been identified.

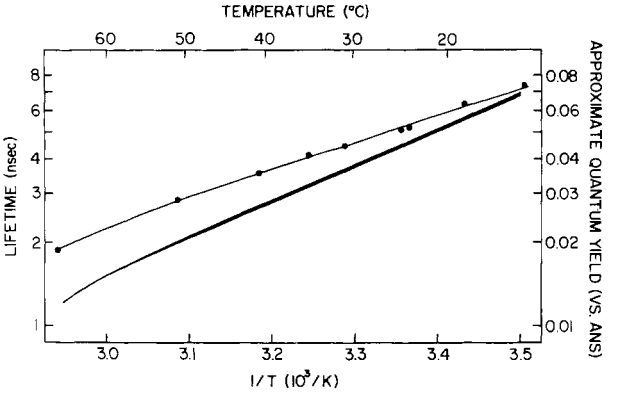

FIGURE 4: Temperature dependence of the fluorescence lifetime (circles) and the fluorescence quantum yield of *cis*-parinaric acid in decane. The absolute scale for the quantum-yield measurements was determined by comparison to ANS at 25 °C. The two lines represent decreasing and increasing temperature scans. The sample was deoxygenated with argon and contained a small amount of BHT. Care was taken to ensure that the spectral shift of the absorption maximum with temperature variation did not affect the relative quantum yield measurements. The sample temperature was controlled with a circulating water bath and monitored with a thermocouple inside the sample cuvette.

An ad hoc partial hypothesis can be formulated concerning the thermal activation of the radiationless process for PnA by noting that a plot of ln k_{nr} vs. $1/T$ is precisely linear. This corresponds to the situation in which

$$k_{nr} = k_{nr}^* e^{-\epsilon/kT} \quad (5)$$

The constants k_{nr}^* and ϵ are interpreted as the radiationless decay rate and excitation energy (above the vibrationless level of the ^1A_g excited state) for a hypothetical radiationless decay path. This is the form expected if k_{nr}^* is very much greater than the decay rate for the lowest vibrational level of the ex-

cited electronic state and if $\epsilon \gg kT$. The values obtained from the slope and intercept of a plot of ln k_{nr} vs. $1/T$ are $k_{nr}^* = 6(\pm 3) \times 10^{11}$ s^{-1} and $\epsilon = 1700$ (± 50) cm^{-1} or 4.9 kcal. The data used to construct this plot are given in the Supplementary Material. This value of ϵ is comparable to the separation between the ^1A_g and ^1B_u excited states. On the other hand, it is also comparable to the energy separation between the ^1A_g excited state and the energy estimated for the second triplet state of the octatetraene chromophore (Hudson and Kohler, 1974). The value of k_{nr}^* of 6×10^{11} s^{-1} is of interest because it is within one or two orders of magnitude of the rate expected for internal conversion. It has been shown that retinal polyenes have a very rapid radiationless decay route, faster than internal conversion, when excited with wavelengths shorter than about 430 nm (Christensen and Kohler, 1974; Waddell et al., 1973; Becker et al., 1971). It is therefore possible that polyenes, in general, have a channel for rapid radiationless decay and that the differences which result in wavelength-dependent or, apparently, wavelength-independent quantum yield are quantitative rather than qualitative.

Conclusions

The parinaryl polyenes have absorption and emission spectra at wavelengths such that spectroscopic measurements in biological systems are not obscured by intrinsic chromophores. Measurements of the environmental dependence of several spectroscopic parameters of these molecules have been carried out and explained on the basis of a model of polyene spectroscopy in which two excited states with very different oscillator strengths play important roles.

The absorption spectrum and extinction coefficient of parinaric acid is shown to be simply and sensitively related to the environmental polarizability. The fluorescence emission spectrum is essentially unaffected by the probe's environment. However, the fluorescence lifetime and quantum yield are strongly affected. In general, the fluorescence lifetime and quantum yield appear to change to nearly the same degree with environmental changes so that the nonradiative rate constant is roughly constant on the order of 10^7/s, which corresponds to an intrinsic lifetime of 100 ns. The temperature dependence of lifetime and quantum yield is nearly linear when plotted as the logarithm of the lifetime (or quantum yield) vs. reciprocal temperature. Deviations from this linear behavior occur in response to lipid-phase transitions.

In conclusion, the features of parinaric acid spectroscopy, which are relevant to its use as a membrane probe, can be understood on the basis of the excited-state order common to linear conjugated polyenes and solvent effects dominated by the polarizability of the solvent.

Acknowledgments

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Supplementary Material Available: Absorption and fluorescence data and ν-a correlations (5 pages). For ordering information consult any current masthead page.

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Conjugated Polyene Fatty Acids as Fluorescent Probes: Synthetic Phospholipid Membrane Studies†

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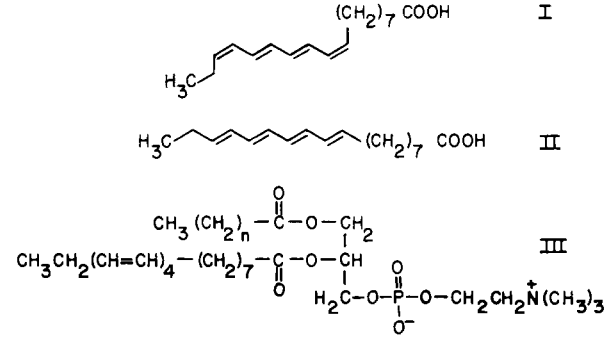
ABSTRACT: The preparation of polyene fatty acid membrane probes *cis*- and *trans*-parinaric acid and parinaroylphosphatidylcholines and their use in studies of several one- and two-component lipid systems are described. The fluorescence quantum yield of *trans*-parinaric acid in dipalmitoylphosphatidylcholine at 20 °C is approximately 0.3; the quantum yield in aqueous solution is negligibly small. Thermal-phase transitions in single-component phospholipid dispersions are monitored with absorption and fluorescence excitation peak position, fluorescence intensity, lifetime, and polarization. The transition temperatures observed are consistent with previous determinations. Shifts in the absorption peak position are related to the bilayer expansion as it undergoes the gel to liquid-crystalline transition, while fluorescence depolarization provides semiquantitative information concerning molecular motion of the probe in the bilayer. A long fluorescence lifetime component is observed for parinaric acid in the solid phase (up to 50 ns), and a short lifetime component is observed (ca. 5 ns) in the fluid phase of dipalmitoylphosphatidylcholine; both lifetime components are observed in the transition region. In most phospholipids, *cis*-parinaric acid detects the melting transition at about 1 °C lower than *trans*-parinaric acid. Partitioning experiments involving mixed populations of phospholipid vesicles show that *trans*-parinaric acid preferentially associates with solid-phase lipids, while *cis*-parinaric acid shows a more equal distribution between solid and fluid lipids. The binding of *cis*-parinaric acid to dipalmitoylphosphatidylcholine at 25 °C is described as a partitioning of parinaric acid between lipid vesicles and the aqueous phase with a partition coefficient of 5×10^5. Several rates are observed in the binding process which are interpreted as rapid outer monolayer uptake and a much slower process of interlamellar exchange. The phase diagram of the binary lipid mixture dipalmitoylphosphatidylcholine–dipalmitoylphosphatidylethanolamine has also been examined and found to be essentially identical to the one constructed using a nitroxide probe.

In the previous paper of this issue (Sklar et al., 1977), the spectral properties of the isomers of the linear polyene fatty acid, parinaric acid, were described. In this paper, the preparation of these probes (I, II, and III) and their spectral properties in model lipid systems are described. The behavior of various spectral parameters of the probes is related to the considerable wealth of information about bilayer structures derived from other techniques.

Materials and Methods

Polyene Fatty Acids. Parinaric acid (9,11,13,15-octadecatetraeneoic acid, PnA[1]; commercially available from Molecular Probes, Roseville, Minn.) was first isolated in 1933 from *Parinari laurinum*[2] (Eckey, 1954). The seed kernel is about 15% oil and nearly 60% of the fatty acid component is parinaric acid (Riley, 1950). About 3 g of PnA can be obtained from a single seed using the procedure described below. The natural product (I), known as α-parinaric acid, was identified as the cis,trans,trans,cis isomer by Gunstone and Subbarao (1967). β-Parinaric acid (II) obtained by treatment of α-parinaric acid with iodine has the all-trans configuration



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[1] Abbreviations used are: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; BHT, 2,6-di-*tert*-butyl-4-methylphenol; PnA, parinaric acid; *cis*-PnA, α-parinaric acid; *trans*-PnA, β-parinaric acid; Me_2DPPE, *N,N*-dimethyl-DPPE; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; TLC, thin-layer chromatography; IR, infrared; UV, ultraviolet.

[2] Most references use the species name *Parinarium laurinum* (Riley, 1950, Gunstone and Subbarao, 1967). According to the botanical literature this is equivalent to *Parinarium glaberrimum* (Kramer, 1951) and the modern name is *Parinari glaberrimum* (Backer and Bakhuizen Van den Brink, 1963). For general reviews of the occurrence and chemistry of fatty acids with conjugated unsaturation, see Hopkins (1972) and Solodovnik (1967).