Anisotropy Decay Associated Fluorescence Spectra and Analysis of Rotational Heterogeneity. 2. 1,6-Diphenyl-1,3,5-hexatriene in Lipid Bilayers†

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ABSTRACT: The application of a new spectroscopic tool [Knutson, J. R., Davenport, L., & Brand, L. (1986) *Biochemistry* (preceding paper in this issue)] for studying rotational microheterogeneity of probe location in lipid bilayer systems is described. Anisotropy decay associated spectra are derived from experimentally obtained polarized emission components. "Early" difference spectra ($I_V - I_H$) contain contributions from both fast and slow rotors, while "late" difference spectra predominantly reflect the emission from slowly rotating fluorophores. Anisotropy decay associated spectra have been used to resolve the emission spectra of 1,6-diphenyl-1,3,5-hexatriene (DPH) imbedded within a known rotationally heterogeneous mixture of two vesicle types (L-α-dimyristoyllecithin and L-α-dipalmitoyllecithin). At 29 °C, diphenylhexatriene within pure dimyristoyllecithin vesicles rotates rapidly, with a small r_∞, while diphenylhexatriene in dipalmitoyllecithin vesicles exhibits a large r_∞. Spectra for diphenylhexatriene imbedded in the two vesicle types show small but significant spectral differences. A spectrum of a mixture of the two vesicle types with DPH lies between these characteristic component spectra. The spectrum extracted for "immobilized" probes in the mixture correctly overlays the dipalmitoyllecithin spectrum. Further studies have shown that diphenylhexatriene exhibits more than one emission anisotropy decay associated spectrum in vesicles of a single lipid type, when that lipid is near its phase transition temperature. Diphenylhexatriene apparently inhabits more than one rotational environment even in these "homogeneous" vesicle preparations.

Fluorescence polarization measurements are frequently used to study the fluidity of a wide range of bilayer membranes (Shinitzky & Inbar, 1974; Glatz, 1978; Haggerty et al., 1978; Martin & Thompson, 1978; Pessin et al., 1978; Bisby et al., 1981; Johnson, 1981; Meaks et al., 1981). The fluidity of the hydrocarbon interior of bilayer membranes has been described in terms of apparent "microviscosity" by comparing the steady-state fluorescence polarization of probes such as 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] in reference oils of known viscosity with that found for membrane systems (Shinitzky et al., 1971; Shinitzky & Inbar, 1974).

Nanosecond time-resolved emission anisotropy studies of DPH in model membrane systems have revealed that in contrast to its rotational behavior in oils, the anisotropy at long times (r_∞) does not always decay to zero (Chen et al., 1977; Dale et al., 1977; Kawato et al., 1977, 1978; Lakowicz et al., 1979; Kinosita et al., 1981). Evidence for anisotropic rotational behavior of probes in bilayer membranes has been obtained by many workers (Hildenbrand & Nicolau, 1979; Parola et al., 1979; Sene et al., 1978; Glatz, 1978; Lakowicz & Knutson, 1980; Knutson et al., 1981; Kowalczyk et al., 1981; Brand et al., 1985). Although the finding of nonzero values for r_∞ raises doubts about interpreting fluorescence polarization data in terms of microviscosity, the measurement has provided useful information for correlations of membrane structure with function (Shinitzky & Inbar, 1974, 1976; Shinitzky et al., 1976; Yuli et al., 1981; Cavalieri et al., 1984; Galeotti et al., 1984).

A number of general and specific theories have been proposed to explain the residual emission anisotropy. The r_∞ term has been described in terms of models involving a homogeneous distribution of probes. These include the general formulations of Heyn (1979) and Jähnig (1979) which identified r_∞ with the order parameter S. Lipari and Szabo (1980) and Zannoni (1979, 1983) developed models from first principles of angular distributions and symmetry. Kinosita et al. (1977, 1982) thoroughly characterized a specific ("wobbling-in-a-cone") model for the rotational behavior of DPH in bilayers. These mathematical formalisms do not in themselves require homogeneity. Since their inception, it has been common to think only of homogeneous probe distributions.

The residual anisotropy term (r_∞) can be described in terms of a heterogeneous model. As an example, two probe populations may coexist, broadly described as "mobile" and "immobile" subensembles. Both rotational models were considered by Dale et al. (1977).

The following describes a method used for evaluating rotational heterogeneity in the emission from a fluorophore. The technique is accomplished via association of a particular probe subfraction, exhibiting a characteristic emission spectrum, with a particular rotational rate. The method has previously been evaluated (Knutson et al., 1986) and here is applied to the DPH/vesicle case, described above, which is usually considered to be a rotationally homogeneous system. Preliminary accounts of this work have been presented (Davenport et al., 1982; Knutson et al., 1982b; Brand et al., 1985).

Theory

Suppose one wishes to study the polarized decay of a simple rotationally heterogeneous system. Further suppose that the *total* fluorescence decay surface, $F(\lambda,t)$, does not resolve into the different components. The difference decay surface (which can be made up either of a time series of difference TRES or equivalently of a wavelength series of difference decays) is

$$\bar{y}(\lambda,t) = \sum \alpha_i(\lambda) y_i(t) \quad (1a)$$

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[1] Abbreviations: ADAS, anisotropy decay associated spectra; DML, L-α-dimyristoyllecithin; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPL, L-α-dipalmitoyllecithin; TLC, thin-layer chromatography; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; TRES, time-resolved emission spectra.

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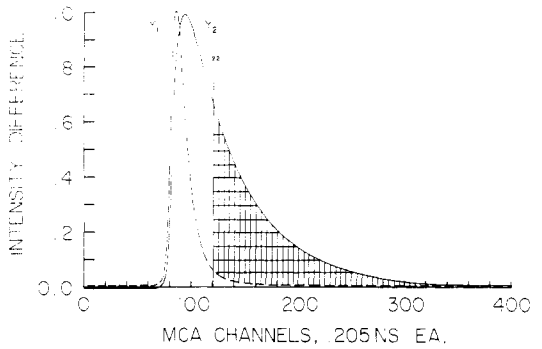

FIGURE 1: Procedure for the determination of the time windows $a \rightarrow b$ and $c \rightarrow d$ used to extract ADAS from time-resolved polarized emission spectra. The time-resolved emission anisotropy for DPH incorporated into a mixture of separately labeled DML and DPL vesicles (1:1 mixture) is defined by a biexponential decay law with one correlation time of ~2 ns and a limiting anisotropy term (r_∞). Y_1 is the synthesized difference decay profile attributed to the faster rotating species. Similarly, Y_2 is the synthesized decay of the difference of the polarized intensities attributed to the immobile fraction. Time windows were chosen (0 → 122 and 123 → 400) such that the late time-gated difference (ADAS) derived from experimentally obtained polarized emission spectra $[GI_{VV}(t) - I_{HV}(t)]$ contains emitted photons from the more slowly rotating species only. Each channel spans 0.205 ns. Excitation was at 355 nm and emission at 430 nm. The temperature was 29 °C.

where

$$y_i(t) = d(t)r_i(t) \tag{1b}$$

and

$$r_i(t) = r_{0_i}e^{-t/\phi_i} \tag{1c}$$

$$d(t) = e^{-t/\tau} \quad \text{or} \quad d(t) = \sum_j f_j e^{-t/\tau_j} \qquad (\textstyle\sum f_j = 1) \tag{1d}$$

Notice that the total decay function, $d(t)$, whether mono- or biexponential, is species (i) invariant and that the overall relationship is separable. Thus, it may be possible to resolve spectra (anisotropy decay associated spectra; ADAS) for the fluorophores in two subpopulations that have equivalent decay times (τ) but different rotational environments. It is of interest to note that anisotropy decay associated spectra may also be obtained for the associative case, where a fluorophore exhibits two or more well-resolved decay times together with two or more well-resolved rotational correlation times [see Knutson et al. (1986)].

Separation of anisotropy decay associated spectra is qualitatively identical with resolution of decay-associated spectra described in detail previously (Knutson et al., 1982a). In practical cases, an emission time "slice" in convolved space (e.g., from $t' = a$ to $t' = b$) is observed (Figure 1). The emission is a mixture of the constituent spectra $\alpha_i(\lambda)$ with mixing coefficients C_i. The correct mixing coefficients are integrals from a to b of the convolved difference decay components Y_1 and Y_2. Here Y_1 and Y_2 are formed from the convolution of y_i with the lamp $L(t)$:

$$\bar{Y}(\lambda,t') = \sum_i \left[\alpha_i(\lambda)\int_0^{t'} L(t)y_i(t'-t)\,dt\right] = \sum_i [\alpha_i(\lambda)Y_i(t')] \tag{2}$$

The mixed difference spectrum in the time slice a to b is defined

$$\bar{Y}(\lambda,a\rightarrow b) = \sum_i \alpha_i(\lambda)C_i(a\rightarrow b) \tag{3a}$$

where

$$C_i(a\rightarrow b) = \int_a^b Y_i(t')\,dt' \tag{3b}$$

Here $\bar{Y}(\lambda,a\rightarrow b)$ is the difference spectrum obtained by summing all photons between the instrument time channels $a \rightarrow b$, and $C_i(a\rightarrow b)$ is the time-dependent mixing coefficient for component $\alpha_i(\lambda)$. Time-resolved polarized emission spectra obtained over two different time windows, $a \rightarrow b$ and $c \rightarrow d$, provide sufficient information to resolve two anisotropy decay associated spectra. The time-resolved difference spectra derived from slices $a \rightarrow b$ and $c \rightarrow d$ are defined

$$\bar{Y}(\lambda,a\rightarrow b) = \alpha_1(\lambda)C_1(a\rightarrow b) + \alpha_2(\lambda)C_2(a\rightarrow b) \tag{4}$$

$$\bar{Y}(\lambda,c\rightarrow d) = \alpha_1(\lambda)C_1(c\rightarrow d) + \alpha_2(\lambda)C_2(c\rightarrow d) \tag{5}$$

These time-resolved difference spectra contain contributions from each emitting species, but in different proportions. The mixing coefficients $C_1(a\rightarrow b)$ and $C_2(a\rightarrow b)$ are just the areas under the curves Y_1 and Y_2 over the time interval $a \rightarrow b$. The coefficients $C_1(c\rightarrow d)$ and $C_2(c\rightarrow d)$ are obtained in a corresponding manner. Since the time-resolved polarized emission spectra are obtained in convolved space, it is also necessary to obtain the mixing coefficients in a convolved space.

Equations 4 and 5 can now be solved for the two unknown anisotropy decay associated spectra, in a manner analogous to that used for resolving decay-associated spectra (Knutson et al., 1982a):

$$\alpha_1(\lambda) = \left\{\left[\frac{C_2(c\rightarrow d)}{C_2(a\rightarrow b)}\right]\bar{Y}(\lambda,a\rightarrow b) - \bar{Y}(\lambda,c\rightarrow d)\right\} \Big/ K_1 \tag{6}$$

$$\alpha_2(\lambda) = \left\{\left[\frac{C_1(c\rightarrow d)}{C_1(a\rightarrow b)}\right]\bar{Y}(\lambda,a\rightarrow b) - \bar{Y}(\lambda,c\rightarrow d)\right\} \Big/ K_2 \tag{7}$$

where

$$K_1 = \left[\frac{C_2(c\rightarrow d)}{C_2(a\rightarrow b)}\right]C_1(a\rightarrow b) - C_1(c\rightarrow d) \tag{8}$$

and

$$K_2 = \left[\frac{C_1(c\rightarrow d)}{C_1(a\rightarrow b)}\right]C_2(a\rightarrow b) - C_2(c\rightarrow d) \tag{9}$$

In the following vesicle studies, resolution of ADAS was even further simplified. A time gate was chosen such that the "late"time window contains primarily photon contributions from the more slowly rotating fluorophore fraction. Hence, the late difference spectrum is directly associated with an immobile probe fraction. This may be easily rationalized by inserting $C_1(c\rightarrow d) = 0$ in eq 7 and 9.

Experimental Procedures

Materials and Methods. L-α-Dimyristoyllecithin (DML) and L-α-dipalmitoyllecithin (DPL) were purchased from Sigma Chemical Co. and used without further purification. Purity was judged by single-spot TLC analysis, using silica gel (Eastman Chromatogram) plates and chloroform/methanol/water (65:25:4 v/v) as the developing solvent. Spot identification was by the phosphorus staining reagent (Dittmer & Lester, 1964). DPH was purchased from Sigma Chemical Co. and used without further purification.

DML and DPL vesicles were prepared by sonication in 0.01 M Tris·HCl buffer (Sigma Chemical Co.) containing 0.1 M NaCl, pH 8.5, at 30 and 50 °C, respectively, well above the phospholipid phase transition temperature. The lipid samples were flushed continuously with argon to minimize any lipid peroxidation (Hauser et al., 1971; Brunner et al., 1976). Separation of single bilayer vesicles was achieved by ultracentrifugation (Beckman Airfuge) at 100000g for 60 min at 4 °C, essentially according to the method of Barenholz et al. (1977). The uppermost fraction of the supernatant was isolated. Lipid phosphorus was determined according to the method of McClare (1971). Total phospholipid concentrations used for fluorescence measurements were typically 0.3 mM.

Labeling of vesicles with DPH was carried out by direct organic solvent injection as described by Chen et al. (1977), giving a final probe to lipid ratio of 1:500. Vesicles were allowed to incubate for 2 h to allow maximum absorption of the dye. The small volume of tetrahydrofuran added during the labeling process was removed by evaporation using argon. For the ADAS studies, mixed vesicle populations of separately labeled DML and DPL bilayers (1 to 1 molar ratio by phosphorus) were prepared. Samples of cosonicated DML and DPL single bilayer vesicles (1 to 1) were also prepared as a control to assess the extent of vesicle fusion of the separately mixed phospholipid vesicle populations.

Fluorescence Spectroscopy. Steady-state fluorescence measurements were performed with a Perkin-Elmer MPF4 fluorometer, as described in detail elsewhere (Chen et al., 1977). A quarter-wave plate was used in the excitation path to obviate any polarization bias. Total intensity emissions were collected by using a "magic-angle" polarizer oriented in the emission path at ~55° to the horizontal axis (~35° to the vertical; Almgren, 1968; Spencer & Weber, 1970). Steady-state emission anisotropies were measured by using Polaroid dichroic film. Excitation and emission wavelengths were 355 and 430 nm, respectively, with excitation and emission bandwidths of 14 and 7 nm, respectively. For all fluorescence measurements, the absorbance of the samples, at the wavelength of excitation, was less than 0.1 in order to obviate inner filter effects (Parker & Rees, 1962).

Nanosecond time-resolved emission anisotropy measurements were performed as described in detail by Chen et al. (1977). Orthogonally polarized decay curves were collected contemporaneously along with the excitation profile in a single photon counting delayed coincidence fluorometer (Badea & Brand, 1979). Excitation of DPH-labeled samples at 355 nm was accomplished by using a Baird Atomic interference filter. Emission wavelengths were selected by using a Bausch and Lomb 0.5-m monochromator, operated under computer control. Excitation and emission bandwidths of 14 and 7 nm were used, respectively, for lipid samples.

Corrections were made for the energy dependence of the photomultiplier transit time (Lewis et al., 1973; Wahl et al., 1974) as previously described (Badea & Brand, 1979).

Collection of Anisotropy Decay Associated Spectra. Time-windowed polarized emission spectra were collected as described in detail elsewhere (Knutson et al., 1982a). Essentially, a "time window" is established by summing the photon counts over a given group of channels on the time axis, occurring after the lamp flash. Orthogonally polarized $I_{VV}(t)$ and $I_{VH}(t)$ decay curves were collected semisimultaneously as a function of wavelength, by stepping the emission monochromator through the emission wavelength region of interest. The photons collected within a given time window were summed and kept within computer memory. Difference spectra $[GI_{VV}(t) - I_{HV}(t)]$ were generated for each time window by simple computer manipulation of the data and plotted as a function of emission wavelength. The value of the G factor correction (Chen & Bowman, 1965) was close to unity across the emssion wavelength region and was determined by using an isotropic standard of 9-cyanoanthracene in ethanol, under identical experimental conditions. Anisotropy decay associated spectra for the DPH-labeled samples were obtained by using identical excitation and emission conditions as outlined above.

Results

Initial experiments were carried out to verify the ability of ADAS to resolve spectra of DPH in a mixture of two different rotational environments. To provide heterogeneity, a mixture of two synthetic phosphatidylcholines with different chain lengths was selected. Figure 2 shows the emission anisotropy for DPH incorporated into small unilamellar vesicles (SUV) of DML and DPL. At 29 °C, the former is above its phase transition temperature near 23 °C (Chapman, 1972) and therefore provides a fluid environment for DPH. In contrast, DPL is below its reported phase transition of near 40 °C (Estep et al., 1978; Lelkes et al., 1979) and restricts the motions of DPH to a greater degree. Clearly a mixture of these vesicles will provide a heterogeneous rotational environment for DPH. The emission anisotropy of a one to one (by phosphorus concentration) mixture of DML and DPL single unilamellar vesicles is presented in Figure 3.

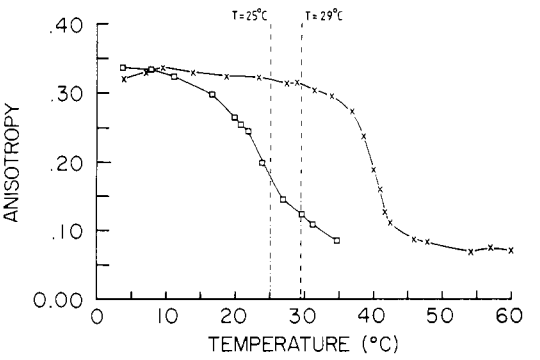

FIGURE 2: Steady-state emission anisotropy as a function of temperature for DPH incorporated into (□) DML vesicles and (×) DPL vesicles. The probe to phospholipid molar labeling ratio was 1:500 with excitation at 355 nm and emission at 430 nm. Excitation and emission bandwidths were both 4 nm.

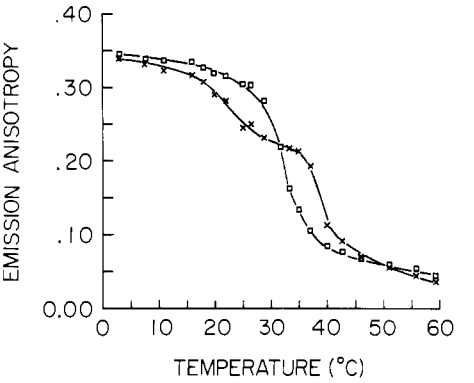

FIGURE 3: Steady-state emission anisotropy as a function of temperature for DPH incorporated into DML/DPL (1:1 molar, by phosphorus) single bilayer vesicles: (×) prepared by cosonication of the separate phospholipids and labeled, and (□) formed by separately mixing DPH-labeled DML and DPH-labeled DPL vesicles (1:1 by phosphorus concentration). The probe to phospholipid molar labeling ratio was 1:500. Excitation was at 355 nm and emission at 430 nm. Excitation and emission bandwidths were both 4 nm.

The transition curve provides evidence that the vesicles retain their individual character when combined. The mixture reveals a well-defined biphasic profile, with sharp inflections in the anisotropy values at the precise phase transition temperatures expected. As a control, DML/DPL single bilayer vesicles were prepared by cosonication of the individual phospholipids and labeled with DPH. Such a preparation, under the same experimental conditions, reveal a broad phase transition change occurring around 32 °C. This compares favorably with similar results obtained previously by Lentz et al. (1976).

The impulse response (deconvolved decay curves) of the emission anisotropy for DPH in DML vesicles, in DPL vesicles, and in a mixutre of DML/DPL vesicles at 29 °C is shown in Figure 4. The anisotropy was adequately analyzed for a single exponential plus a constant term (r_∞). The r_∞ value was high (~0.3) for DPH in DPL vesicles where the probe is located in a rather rigid matrix. The r_∞ term was much lower (0.05)

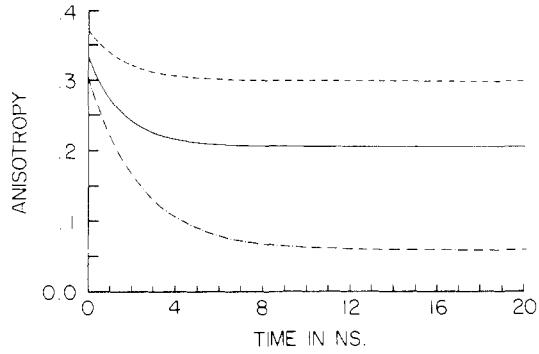

FIGURE 4: Impulse response functions (deconvolved) for the decay of the emission anisotropy, $r(t)$, of DPH-labeled DPL vesicles (- - -) and DML vesicles (—·—) and for a 1:1 separately labeled DML/DPL vesicle mixture (—) at 29 °C. The parameters obtained for $d(t)$ for DPH/DPL vesicles best fit a double exponential decay function (f_1 = 0.119, τ_1 = 3.82 ns, f_2 = 0.881, τ_2 = 10.41 ns, and χ^2 = 1.83). Similarly for DPH/DML vesicles, f_1 = 0.057, τ_1 = 4.18 ns, f_2 = 0.943, τ_2 = 8.47 ns, and χ^2 = 1.76, and for the DPH/mixed vesicle system, f_1 = 0.233, τ_1 = 5.46 ns, f_2 = 0.767, τ_2 = 10.26 ns, and χ^2 = 2.34. The obtained parameters for $r(t)$ for DPH/DPL vesicles were recovered in terms of a single exponential decay plus a constant (β = 0.07, ϕ = 1.63 ns, r_∞ = 0.30, and χ^2 = 1.09). Similarly for DPH/DML vesicles, β = 0.25, ϕ = 2.35 ns, r_∞ = 0.06, and χ^2 = 1.12, and for DPH/mixed vesicles, β = 0.13, ϕ = 1.56 ns, r_∞ = 0.21, and χ^2 = 1.53. Excitation was at 355 nm and emission at 430 nm with bandwidths of 14 and 7 nm, respectively. The molar labeling ratio was 1:500 (probe to phospholipid). Values for the steady-state emission anisotropy for DPH/DPL, DPH/DML, and DPH/mix were 0.30, 0.12, and 0.23, respectively.

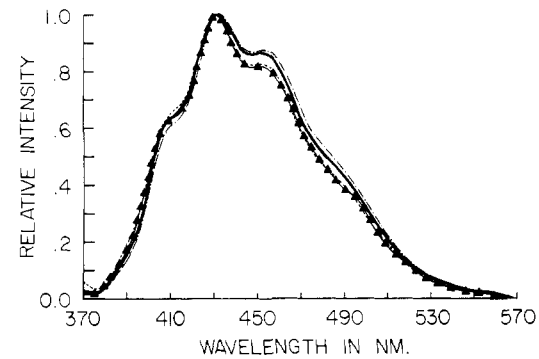

FIGURE 5: Anisotropy decay associated spectra for a DPH-labeled 1:1 mixture of DML and DPL vesicle preparations at 29 °C. The steady-state emission spectra for the individual (- - -) DPL and (—·—) DML vesicles and for the 1:1 vesicle mixture (—) are shown. The anisotropy decay associated spectrum (Δ) corresponding to the immobile probe fraction shows superimposition with the DPL steady-state spectrum. Excitation was at 355 nm with excitation and emission bandwidths of 14 and 9.9 nm, respectively.

for the probe in DML vesicles. The r_∞ for the mixture is intermediate in value. The mixed anisotropy was divided into two functions, one describing the averaged "free" rotation (fast, ϕ < 3 ns) and the other for immobile ($\phi \rightarrow \infty$, r_∞) probes. These $r(t)$ functions, each multiplied by the total decay function, were reconvolved to obtain mixing coefficients as described above. The former (fast rotation) areas were small in early windows and negligible in late windows. Thus, the immobile ADAS is obtained from the late difference TRES (minus a negligible multiple of the early TRES). The mobile ADAS is only a small fraction of the total signal and therefore noisy. For precise isolation of this spectrum, it will be necessary to focus on a different admixture or to collect many photon counts.

The peak-normalized total intensity fluorescence spectra obtained for the individually labeled vesicle samples are shown in Figure 5. DPH in the DML and DPL vesicles exhibits small but significant vibrational differences (Zannoni et al., 1983). In particular, at longer wavelengths, DPH in DML vesicles exhibits a relatively larger intensity compared to that in DPL vesicles. The steady-state emission spectrum for the vesicle mixture (solid line) contains contributions from DPH imbedded in both vesicle types. As expected, the immobile ADAS compares favorably with the steady-state spectrum obtained for DPH in DPL vesicles, the more immobile probe fraction. An unlabeled vesicle preparation exhibited a small scatter component around 400 nm. This spectral blank was insignificant in the region of interest. Since the instrumental or G factor (Chen & Bowman, 1965) correction was found to be close to unity across the emission wavelength region, the anisotropy decay associated spectra derived are not distorted by fluctuations in this value. The technique of ADAS still remains tenable if the G factor varies with emission wavelength, so long as this correction factor is time invariant.

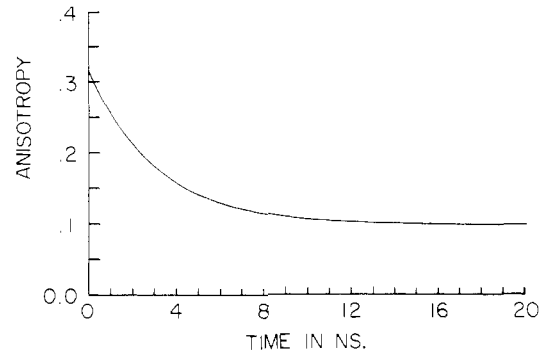

FIGURE 6: Impulse response function (deconvolved) for the decay of the emission anisotropy $r(t)$ of DPH-labeled DML vesicles at 25 °C. The decay of the total fluorescence emission best fitted a double exponential decay function (f_1 = 0.106, τ_1 = 1.18 ns, f_2 = 0.894, τ_2 = 9.09 ns, and χ^2 = 1.64). The parameters obtained for $r(t)$ were best expressed in terms of a single exponential decay function plus a constant term (β = 0.21, ϕ = 3.03 ns, r_∞ = 0.15, and χ^2 = 1.20). Excitation was at 355 nm and emission was 430 nm, with bandwidths of 14 and 7 nm, respectively. The molar labeling ratio was 1:500 (probe to phospholipid). The value of the steady-state emission anisotropy was 0.20.

Further studies have shown that DPH exhibits more than one emission anisotropy decay associated spectrum in single bilayer vesicles of DML alone. Figure 6 shows the deconvolved time-resolved fluorescence emission anisotropy for DPH in DML vesicles at 25 °C. At this temperature, DML is close to the phospholipid phase transition (see Figure 2), and the bilayer is expected to be composed of both solidlike and fluidlike phases (Sklar et al., 1977; Shimshick & McConnell, 1973). Analysis of the time-dependent anisotropy again shows an initial rapid decrease with time, which appears to level off at an appreciable r_∞ values (close to 0.1). These results confirm those previously obtained by Chen et al. (1977). Similarly, the total fluorescence decay was best expressed as a biexponential function. It was not adequately analyzed as a single exponential, although some vesicle studies have found monoexponential decays (Veatch & Stryer, 1974; Lentz et al., 1976; Kawato et al., 1977; Klausner et al., 1980). The parameters obtained are summarized in the legend to Figure 6.

The time-resolved difference spectra (Figure 7a) for this system obtained from either early or late time-gated polarized decay curves again reveal varied ratios between the vibrational peaks, with trends identical with those observed for the mixed vesicle system. The anisotropy decay associated spectrum corresponding to the immobile probe fraction is of lower intensity on the red edge. Thus, it appears that DPH inhabits more than one rotational environment in these vesicles. Figure 7b shows the corresponding early and late time-gated total intensity ($V + 2H$) spectra. With the particular choice of time

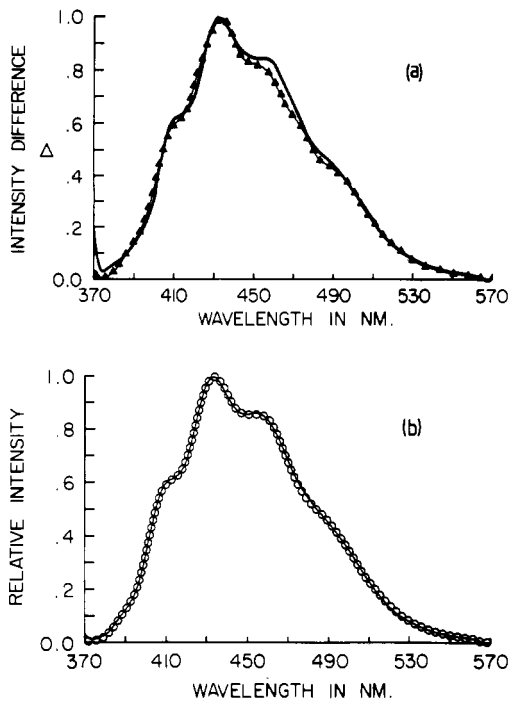

FIGURE 7: (a) Polarized difference TRES for a DPH-labeled DML vesicle sample at 25 °C. The late difference spectrum (▲) directly represents the ADAS of the immobile probe fraction in this system. The early difference (—) includes contributions from both mobile and immobile probes. Excitation was at 355 nm, with excitation and emission bandwidths of 14 and 9.9 nm, respectively. (b) Total intensity TRES obtained by using identical time-gating conditions and for the same DPH-labeled, DML vesicle sample at 25 °C. The early and late total intensity spectra ($V + 2H$) show complete superimposition. Excitation was at 355 nm with excitation and emission bandwidths of 14 and 9.9 nm, respectively.

windows chosen for the ADAS experiment, it can be seen that the spectra are not well resolved by lifetime association. Such results provide evidence for nonassociative behavior (Chen et al., 1977; Beechem et al., 1984) between fluorescence lifetimes and rotational correlation times, under these conditions.

Discussion

The hindered rotation of DPH imbedded within lipid bilayers is a well-known phenomenon. The variety of models proposed to explain the nonzero r_∞ value have provided a solid mathematical framework for anisotropy decay. Each of these models described a homogeneous ensemble of probes that explain the presence of r_∞; i.e., each probe experiences the same mean environment and the same angular barriers to complete rotation. All of these models could easily be extended to the coexistence of two different populations, having (e.g.) different "cone angles" or (more general) order parameters. Dale et al. (1977) recognized the key role that microheterogeneity could play and sought to associate the total and anisotropy decay functions. The lack of clear $\tau \leftrightarrow \phi$ association at that time prevented these workers from discerning homogeneous from heterogeneous origins of r_∞, and they suggested that the system ought to contain elements of each. The heterogeneous ordering concept has been largely set aside since then.

In systems where both P_2 and P_4 distribution parameters can be derived, evidence has recently been obtained (Kooyman et al., 1983; Voss et al., 1983; Van de Meer et al., 1984; Ameloot et al., 1984) for the presence of a significant population of probes lying perpendicular to the membrane director (e.g., molecules in the plane). This interpretation, while dependent on the particular homogeneous distribution assumed, provides for the consideration of probes "outside" the presumed barriers (Andrich & Vanderkooi, 1976).

In this contribution, we present evidence that probes which remain aligned well after excitation exhibit a different emission spectrum. These data are not consistent with a simple homogeneous ordering process. The extent of heterogeneity is unknown at this time; the simplest (though least likely) extreme is that of freely rotating probes (found in "fluid" regions) in equilibrium with a set of rigidly oriented fluorophores (in "gel" domains). A more intermediate model (mixtures of highly restricted and weakly ordered populations) is probably more correct. Perhaps *spectral* observations in oriented systems, even though complicated by wavelength-dependent interface transmission properties, can provide a fuller understanding.

Orientation-dependent quenching, such as that provided by energy transfer, may provide another experimental "window" into this process. A heterogeneous distribution of DPH molecules across the bilayer has been observed by using energy transfer (Davenport, 1981; Davenport et al., 1985). External perturbations or biochemical variations that alter the quantity of probe in each ensemble may also help to overdetermine the order parameters. A particularly promising approach is that of pressure change. Chong and Weber (1983) have observed spectral changes similar to those presented here, when DPH-labeled DML vesicles were isothermally subjected to 1 kbar. They propose that at increased pressure, the probe in DML vesicles is preferentially distributed into more "solid" regions.

Associative behavior (between total decay and anisotropy decay) may now be resolved with global analysis tools (Beechem et al., 1984; Brand et al., 1985). An association weaker than the one to one correspondence of lifetimes with correlation times, such as found for parinaric acid (Wolber & Hudson, 1982), may exist for DPH. In this case, nonglobal associative fits are difficult, whereas global analyses (across emission and excitation bands) hold more promise.

In summary, we propose two main concepts: (1) that the ordering of DPH in lipid bilayers has heterogeneous origins and (2) that ADAS may be used to resolve the spectra of differently rotating fluorophores in biochemical systems.

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