MEMBRANE STRUCTURAL DOMAINS

Resolution Limits Using Diphenylhexatriene Fluorescence Decay

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ABSTRACT Measurement of multiple fluorescence decay times of 1,6-diphenyl-1,3,5-hexatriene (DPH) in membranes can in principle be used to investigate structural domains of lipid bilayers. To assess the feasibiilty of this approach using phase and modulation techniques, we reduced experimental errors specifically associated with performing these measurements on membrane suspensions (probe self-quenching, background fluorescence, turbidity-induced artifacts) and determined empirically the level of precision thereby obtainable. Next we used these precision limits in theoretical calculations to conclude that the ratio of two coexisting decay times must exceed 1.3 if they are to be resolved with reliable accuracy. To demonstrate that such resolutions could be accomplished experimentally in membrane suspensions, three approaches were taken. First, the fluorescence decay of aqueous quinine sulfate quenched by chloride ion was resolved from that of membrane-associated DPH as long as the lifetime ratios of these two fluorophores exceeded the predicted value. Second, populations of DPH-containing lipid vesicles with single (or nearly single) decay times were mixed together, and when there were only two major lifetime components that differed by more than 30%, the resulting heterogeneous fluorescence could be resolved into the two expected lifetime components. Finally, DPH fluorescence decay measurements were correlated with phase behavior in well-characterized lipid systems, revealing a short lifetime component of DPH fluorescence associated with gel-phase lipid vesicles. From these studies, we conclude that only in special cases can co-existing gel and fluid phases be resolved by means of DPH lifetime heterogeneity, within the limits of precision defined herein.

INTRODUCTION

The investigation of membranes on a molecular level generally requires indirect techniques. Various fluorescence parameters of 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] have been used to probe membrane structure and lipid dynamics by a great many workers during the last decade (for a comprehensive review, see reference 1). Some of the advantages of studying membrane behavior with DPH include extremely high sensitivity (<1 μmol of membrane lipid is needed for an experiment), very small required concentration of probe relative to lipid (typically ≤1 DPH/500 lipids) so that the overall composition of the membrane is not significantly altered, and the very hydrophobic nature of DPH and its low quantum yield in water, assuring that measured fluorescence emanates from within the membrane and not from the surrounding aqueous environment. In addition, DPH has been shown to align principally with the lipid acyl chains (2), and its fluorescence polarization can be interpreted as reflecting the rate and extent of motion of the acyl chain region in lipid bilayers (3, 4). Further advantages include the well-documented ability of DPH to detect thermodynamic lipid phase boundaries, and the nonpreferential partitioning of DPH into lipid bilayers of different phases or composition (5, 6).

While a great many studies have employed time-averaged or steady-state properties of DPH, far fewer investigations have used the time dependence of DPH fluorescence as a tool for examining membrane properties. This is due both to the requirement for more sophisticated equipment for time-resolved measurements, and to the difficulty of accurately making these measurements in membranes. Despite experimental difficulties, fluorescence decay time measurements can, in principle, be used to detect and even quantitate coexisting regions of different lipid structure (referred to as domains). Several studies (7–9) have applied this method to the problem of resolving such coexisting membrane domains.

[1] *Abbreviations used in this paper:* DPH, 1,6-diphenyl-1,3,5-hexatriene; POPOP, 2,2′-*p*-phenylenebis(5-phenyl)oxazole; egg PC, hen yolk phosphatidylcholine; DMPC, 1,-2-dimyristoyl-3-sn-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-3-sn-phosphatidylcholine; Chol, cholesterol; SR lipid, phospholipids extracted from rabbit sarcoplasmic reticulum; LMVs, large, multilamellar vesicles; REVs, reverse-phase evaporation vesicles; SUVs, small, unilamellar vesicles.

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The construction of phase diagrams as well as freeze-fracture electron microscopy can reveal coexisting regions of apparently different lipid structural organization when conditions are thermodynamically favorable for the lipid bilayer to be in a two-phase state (5, 6). However, situations of potential physical significance may exist in bilayers where a lipid micro-domain might be too small and/or too short lived to be defined thermodynamically or detected by electron microscopy. For example, enhanced ion permeability and dye binding of saturated phosphatidylcholine bilayers near the $P_{\beta}' \leftrightarrow L_{\alpha}$ phase transition (10) has been ascribed to boundaries between small, transient, and structurally distinct lipid regions (11), but this explanation remains speculative.

To use DPH lifetime measurements to investigate coexisting membrane structural domains, it must be possible to accurately resolve multi-exponential fluorescence decays. To accomplish this even in a homogeneous solution, highly precise measurements are required. We have recently shown (12) how precise and accurate measurements in solution can be made with phase and modulation techniques using the commercially available instrument (SLM Instruments, Urbana, IL) developed by Spencer and Weber (13, 14). Specifically, lifetime measurements by phase and modulation require determination of the phase angle and modulation amplitude of the emitted fluorescence relative to the sinusoidally modulated light beam, which excites the fluorescent sample. This is most accurately accomplished using a homogeneous, fluorescent reference solution whose lifetime is closely matched to that of the sample (isochronal standard) (12).

To accurately resolve heterogeneous lifetimes (multi-exponential decays) of DPH in membranes, errors specifically associated with the use of a membrane suspension must be eliminated. Here we consider several systematic errors inherent in measuring DPH lifetimes in membranes and propose experimental procedures to compensate for these errors. In addition, we have generated theoretical data for a variety of hypothetical two-component systems, and investigated the sensitivity of two-component analysis to the errors that we have found inherent in phase and modulation lifetime measurements. We have tested these calculations by experimentally resolving known lifetimes from mixtures of quinine sulfate and DPH-labeled lipid vesicles. Also, we have analyzed two special case mixtures of different DPH-labeled lipid vesicle preparations and experimentally verified the potential accuracy of lifetime resolution by comparison to the DPH lifetimes of the individual lipid preparations. Finally, we have determined the temperature dependence of DPH lifetimes in some simple lipid bilayer systems with well-characterized phase behavior. While we have found single-exponential fluorescence decay for DPH in liquid-crystalline (fluid, high temperature) phase lipid vesicles, lifetime heterogeneity was ubiquitous for gel (solidlike, low temperature) phase lipids. This result severely complicates the interpretation of DPH lifetime heterogeneity in terms of coexisting gel and fluid lipid domains.

MATERIALS AND METHODS

DPH was purchased from Molecular Probes (Junction City, OR), and stock solutions (2 and 10 mM) were stored in acetone, in blackened vials, and at −20°C under argon until use. Isochronal reference fluorophores were obtained as described previously (12). Quinine sulfate solutions were prepared as described elsewhere (15, 16). Hen egg yolk phosphatidylcholine (egg PC) was isolated and purified as described previously (6). Phospholipid extracted from rabbit sarcoplasmic reticulum (SR lipid) was prepared as described previously (17). 1,2-dimyristoyl-3-sn-phosphatidylcholine (DMPC) and 1,2-dipalmitoyl-3-sn-phosphatidylcholine (DPPC) were purchased from Avanti Biochemical (Birmingham, AL) and were judged to be at least 99% pure by thin-layer chromatography on prewashed, silica gel GHL plates with 0.01 M dipotassium oxalate as binder (Analtech, Inc., Newark, DE), eluting with chloroform/methanol/water (65:25:4; vol/vol/vol). The DPPC, however, was initially found to have approximately three times more background fluorescence than other lipids, so the DPPC chloroform stock (0.025 M) was twice filtered over acid-washed charcoal, which reduced its intrinsic fluorescence to the level observed for other lipids. Cholesterol (Chol) was obtained and used as described previously (6). All solvents were either high pressure liquid chromatography or spectrophotometric grade, and water was doubly distilled (initially from alkaline permanganate) in glass and stored under argon at 4°C until use. KCl (Ultrapure, lot No. 2179) was purchased from Heico, Inc. (Delaware Water Gap, PA) and used without further purification.

Lipid Vesicle Preparation

Large, multilamellar vesicles (LMVs) were prepared as previously described (6). Identical results were obtained whether the DPH was co-dried with the lipid, or injected into the preformed vesicles, except that in the latter case a day of swirling above the main lipid phase transition temperature was required for complete incorporation (maximum fluorescence intensity) of the DPH (18).

Large, unilamellar vesicles were prepared by slow dilution and dialysis (above the main lipid phase transition) of an aqueous octylglucoside-lipid suspension by an extension of the procedure of Mimms et al. (19). Details of the preparation, characterization, and phase behavior of these vesicles are presented elsewhere (20). For these vesicles, the DPH was injected (typically 0.5 μl of a 2 mM acetone solution) into the preformed vesicle suspension. Maximum fluorescence intensity of DPH was attained within 3 h.

Reverse-phase evaporation vesicles (REVs) were prepared by a modification of the procedure described by Szoka et al. (21). Typically, 40 μl of a 25 mM lipid stock in $CHCl_3$ was added to 5 ml of 100 mM KCl (in doubly glass-distilled H_2O) in a 10 ml culture tube with a Teflon®-lined screw cap. For fluorescent samples, 0.5 μl of 2 mM DPH in acetone (= 1 DPH/1,000 lipids) could be injected into the suspension with vortexing at this point. The DPH also could be added later to preformed vesicles with identical results. This mixture was then repeatedly (typically 5–10 times) heated to 50°C, vortexed for ~5 s, and a moderate vacuum (450 mmHg) briefly applied to remove the $CHCl_3$, until the formation of bubbles upon application of the vacuum essentially ceased. At this point, a clear suspension had formed, which was centrifuged at 10,000 *g* for 4 min in a microfuge (Beckman Instruments, Inc., Fullerton, CA). The resulting supernatant was extruded sequentially through polycarbonate filters (Nuclepore, Pleasanton, CA) with pore sizes of 0.4, 0.2, and 0.1 μm. Negative staining electron microscopy (using 2% ammonium molybdate; 20) revealed that the vesicles were relatively uniform in size (mostly 1,000–2,000 Å in diameter after extrusion through the 0.1 μM filter). No multilamellar vesicles were seen in any of the microfuged and filtered preparations.

Small, unilamellar vesicles (SUVs) were prepared by sonication and

isolated by ultracentrifugation as described previously (22). Fused, unilamellar vesicles were prepared by prolonged storage of SUVs at 4°C (23). All DPH-labeled vesicles were stored above their phase transition temperatures and in the dark before use.

Fluorescence Measurements

Fluorescence lifetime measurements were made with an SLM 4800 spectrofluorometer (SLM Instruments, Urbana IL) equipped with EMI 9813 photomultipliers (EMI Electronics, Middlesex, United Kingdom) and a modified, multi-temperature cuvette holder (15). The excitation source was the 366-nm line of a 200 W Hg-Xe lamp (Canrad-Hanovia, Newark, NJ) isolated with a 0.5-nm slit on the excitation monochromator in addition to a UG-1 filter (Schott Optical Glass Inc., Duryea, PA). Fluorescence emission was detected without a monochromator, through a 3 mm KV 450 Schott filter (high-pass, 50% transmittance at 450 nm; Schott Optical Glass Inc.). Measurements were made with the excitation polarizer 35° from vertical and the emission polarizer removed from the instrument (14). Measurements with one polarizer (35° from vertical in the excitation beam were indistinguishable from those made with two appropriately oriented (14) polarizers for vesicles in either the fluid or gel phases. However, two polarizers substantially reduced signal intensity and therefore increased uncertainty in the measurements. We do note that DPH phase lifetimes recorded with no polarizers for gel phase vesicles were slightly different (up to 0.2 ns) from those recorded using one or two polarizers, although the two methods of measurement (i.e., with or without polarizers) were indistinguishable for fluid phase vesicles. On-line data acquisition was performed as previously described (12, 15). To characterize lifetime heterogeneity, calculated (Eqs. I.29 of reference 14) and measured apparent lifetimes (three obtained from phase shifts and three from modulation ratios at 6, 18, and 30 MHz modulation frequencies) were compared, and the sum of squares of their differences (χ^2) was minimized to obtain the most likely pair of lifetimes (τ_1 and τ_2) and fractional intensities ($\alpha_1 = 1 - \alpha_2$, α_2) to account for the observed data. Details of this procedure are given elsewhere (12, 14).

Sample lifetimes were measured against one of three isochronal reference standards: DPH in cyclohexane (τ = 8.60 ns), DPH in hexadecane (τ = 9.62 ns), and 9-anthracenecarbonitrile in absolute ethanol (τ = 11.9 ns), whichever was most closely matched to the sample lifetime. The lifetimes of these standards (which were not deoxygenated) were determined (12) at 23°C against a reference standard of POPOP in absolute ethanol (24). The reference standard was maintained at 23°C in one position of our special cuvette holder. Samples held in the other positions could be independently equilibrated (or scanned) at any desired temperature from 0 to 60°C (12, 15). The optical densities of all samples and standards were kept as low and as closely matched as possible, typically at values ≤0.07 (12). Fluorescence intensities of all samples and standards were roughly equal to that of 2×10^{-7} M DPH in cyclohexane.

Blank vesicle samples for each experiment were prepared identically to the labeled vesicles, except for the addition of the DPH. The intrinsic fluorescence of the blanks was measured and compared with the corresponding fluorescent samples containing DPH. When the blank fluorescence intensity was found to be significant (≥0.5% of the sample), fluorescence phase shifts and modulation ratios of the blank were measured under the same conditions as the sample. Using the measured intensity of the blank, each of the six sample lifetime measurements (phase and modulation at 6, 18, and 30 MHz) was individually corrected by generating (using Eqs. I.29 of reference 14) the lifetime expected for the intensity-weighted mixture of the blank lifetime and the observed sample lifetime for that particular measurement. The difference between the lifetime thus generated and the observed sample lifetime provided an estimate of the error in lifetime introduced by the background fluorescence. This approximate lifetime error was then subtracted from the sample lifetime, yielding a corrected sample lifetime. Note that this procedure was applied to each of the individual, observable, apparent lifetimes. This correction procedure involves a slight approximation (related to the nonlinear functionality of the phase and modulation lifetime relations given in reference 14), but is quite valid for the small corrections made here (all blanks had <2% of the corresponding sample intensity).

RESULTS AND DISCUSSION

Sources of Experimental Error in the Measurment of DPH Lifetimes in Membranes

The data in Fig. 1 illustrate several of the problems we have encountered in trying to make accurate phase and modulation lifetime measurements of DPH in membranes. In Fig. 1, we have plotted the apparent lifetimes by phase and modulation at 6, 18, and 30 MHz for varying amounts of DPH in 0.25 mM DPPC REVs at 49°C. The data shown in Fig. 1 have already been corrected for the actual measured lifetimes of the background fluorescence observed in an unlabeled (blank) vesicle sample as described in Materials and Methods. Without such a correction, the phase lifetimes decreased somewhat at high lipid-to-DPH ratio (i.e., as the background fluorescence contribution became more significant; data not shown). Treatment of the background as scattered light (i.e., with zero lifetime) resulted in overcorrection and caused substantially higher phase lifetimes at high lipid-to-DPH ratios (not shown).

An interesting feature of the data in Fig. 1 is the decrease in apparent lifetimes for lipid/DPH ratios below

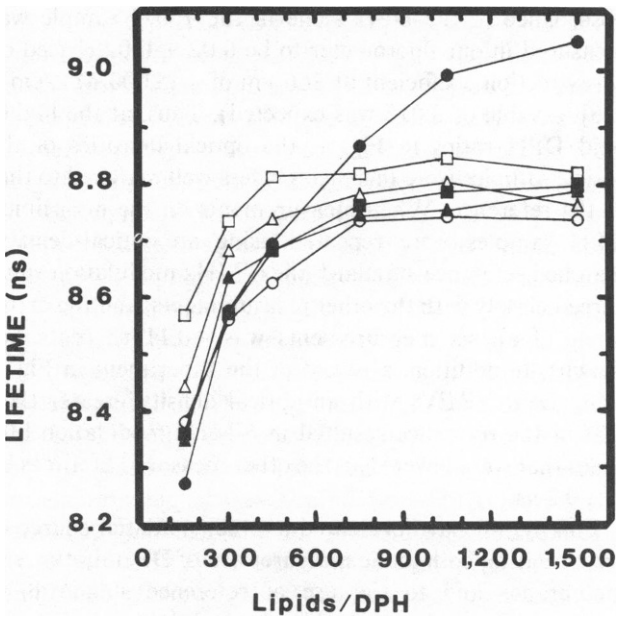

FIGURE 1 Observed fluorescence lifetimes of DPH in 0.25 mM DPPC REVs at 49°C, plotted against the molar ratio of lipid to DPH (Lipids/DPH) in the sample. Lifetimes were obtained from modulation ratio measurements (filled symbols) and phase shift measurements (open symbols) at modulation frequencies of 6 MHz (circles), 18 MHz (triangles), and 30 MHz (squares). The data were corrected for blank fluorescence as described in Materials and Methods.

750/1. This may suggest that DPH molecules self-quench below 750 lipids/DPH. Therefore, we have chosen to use a ratio of 1,000 lipids/DPH to avoid this possible complication. We note that decreased fluorescence lifetimes at lower lipid-to-DPH ratios are not due to contamination by acetone (the solvent used for addition of DPH to vesicle samples), as the addition of even greater amounts of acetone to a 400:1 (lipid/DPH) sample had no significant effect on the observed DPH lifetimes.

Another aspect of Fig. 1 that deserves mention is the high value of the 6-MHz modulation lifetime relative to the other measurements. As previously discussed (12), errors in apparent lifetime can be introduced by failing to match the optical density of the sample and reference solutions. The general effect of unmatched sample and reference optical densities is due to an experimental difficulty characteristic of the differential measurements made in phase fluorometry, namely different imaging patterns on the sample and reference photocathodes. This causes distortion especially of the modulation ratio measurements, since these are most sensitive to signal intensity. This showed up mostly in our 6-MHz modulation data, since, for relatively low lifetimes (<10 ns), small errors in the modulation ratio will be most sensitively detected at low modulation frequencies. For the sake of consistency, all of the measurements in Fig. 1 were made against the same reference standard solution (DPH in cyclohexane), which had an optical density of 0.07. For the preparation of DPPC REVs used for Fig. 1, the optical density at 366 nm of the blank sample was found to be only 0.03, and the absorbance of the DPH alone in the 750/1 sample was measured in our fluorometer to be 0.02 ± 0.02 (based on an extinction coefficient at 366 nm of ~45,000 M^{-1} cm^{-1} [25], a value of 0.015 was expected). Thus, at the higher lipid/DPH ratios in Fig. 1, the optical densities of the vesicle samples were increasingly less well matched to that of the reference. When measurements on the high lipid/DPH samples were repeated using an optical-density-matched reference standard, the 6-MHz modulation value agreed closely with the other measurements, and the entire range of all six measurements was <0.11 ns (data not shown). In addition, a repeat of the experiment in Fig. 1 using DPPC REVs with an optical density greater than that of the reference resulted in 6-MHz modulation lifetimes that were lower than the other measured lifetimes by 0.1–0.2 ns.

Finally, we have evaluated another potential source of error relating both to the measurement of DPH lifetimes in membranes and to the use of reference standards in nonaqueous solutions, namely the possibility that errors could be caused by using a reference standard whose refractive index was significantly different from that of the sample. Assuming that the cause of such an error was simply a time delay due to the reduced velocity of light in a medium of higher refractive index, we calculated the phase lifetime errors (modulation lifetime measurements would be unaffected under this assumption) that might be introduced. In the worst case considered (30-MHz phase lifetime measurement for an aqueous sample measured against a benzene reference), the expected error for a 10-ns lifetime sample was calculated to be only 25 ps. Experimentally, we have never observed appreciable spreading of apparent phase and modulation lifetimes in comparative measurements of homogeneous samples with reference standards of different refractive index, which would argue also against the possibility that refractive index mismatches could cause a significantly different image on the photocathode. For measurements of nanosecond lifetimes, we conclude that refractive index differences between reference and sample may be ignored, although these may be significant in measurement on the 100-ps time scale.

Precision of DPH Lifetime Measurements in Membranes

We have analyzed DPH phase and modulation lifetime data obtained from 10 different DPPC vesicle samples above their main phase transition temperatures. All six measurements (phase and modulation at 6, 18, and 30 MHz) for each sample were approximately the same, indicating that effectively only one lifetime component was present in each sample. By determining the deviation of each measurement from the mean lifetime of the data set for that sample, we were able to estimate the precision (though not the absolute accuracy) of each of these measurements. The mean values over the 10 different samples of these mean deviations were 18 ± 17, 37 ± 39, 41 ± 47 ps (phase shift) and 52 ± 45, 26 ± 14, 32 ± 36 ps (modulation ratio) for 6, 18, and 30 MHz, respectively. This range is quite comparable to the precision of measurements in homogeneous solution (12, 15, 16), demonstrating the effectiveness of our procedures for performing DPH lifetime measurements in membranes.[2] The large standard deviations on these mean errors reflect the fact that on some samples errors in all six measurements were uniformly much larger or much smaller than the means. This probably reflects greater or lesser degrees of success in tuning the Debye-Sears modulation tank on different days.

On the basis of this analysis of deviations, we have assigned error margins (encompassing statistical errors and errors due to instrumental irreproducibility) of 100 ps to the 6-MHz modulation and 30-MHz phase measurements, and 50-ps to the other four measurements (6- and 18- MHz phase, 18- and 30-MHz modulation). Better than 80% of the data from the 10 vesicle samples examined

[2]It should be stressed that the success of our procedures depended principally on the use of isochronal reference standards, without which, imprecise and irreproducible results were common. It is likely that the use of these standards minimizes the proposed (26) systematic errors associated with commercial phase fluorometers (i.e., phototube color effects and light beam modulation inhomogeneities).

fell within these error margins. These error margins, determined for samples with lifetimes of ~8 ns, represent reasonable estimates of our precision limits for sample lifetimes in the range of 7–12 ns, i.e., for any reasonable average lifetime of DPH in a membrane.

Above the main lipid phase transition (e.g., at temperatures >42°C for DPPC) the narrow distribution of apparent lifetimes within each data set supports the contention that the fluorescence decay of DPH in liquid-crystalline lipid bilayers is reasonably well described by a single decay time. In each of ten experiments on vesicles of varying structure and composition, we have found that a single exponential model adequately described the data obtained in the fluid phase ($X^2_{v1}/X^2_{v2} = F_{12} = 0.61$ to 1.13, $P = 0.74$ to 0.58).[3]

The arguments presented here refer principally to statistical errors. Our agreement with other laboratories (see Controversy over DPH Lifetime Heterogeneity) and our success in resolving multiple lifetime systems (see below) argue that we have been successful in eliminating significant systematic errors in the use of the phase fluorometer (SLM 4800, SLM Instruments).

Theoretical Limits of Resolution

We have examined the limitations placed by the observed precision (roughly 0.5–1.0%) on our ability to accurately resolve two coexisting lifetime components. To explore this issue, we used Eqs. I.29 of reference 14 to generate data (three phase shift and three modulation ratio values) for hypothetical values of τ_1, τ_2, and α_2 for two-component systems (τ_1, τ_2 refer to component lifetimes, α_2 to the fractional intensity of component two). The synthesized data were then altered by the observed precision margins according to the two worst distributions of these errors (i.e., so that the greatest possible error in the subsequent heterogeneity analysis would result). In the first method, the error margins were added to the modulation lifetimes and subtracted from the phase lifetimes. The reverse procedure (error margins subtracted from the modulation lifetimes, added to the phase lifetimes) was applied in the second method. The altered data sets were then analyzed (7, 12) in terms of two lifetime components (τ_1 and τ_2) and the fractional fluorescence intensity of component two (α_2, note that $\alpha_1 = 1 - \alpha_2$). These resulting analyzed components could then be compared with the original, hypothetical components.

The results of one group of two-component simulations are depicted in Fig. 2. In this case, the hypothetical components were assumed to be 10 ns (τ_1) and 5 ns (τ_2) as indicated by the horizontal dotted lines, and the assumed fractional intensity was varied, as indicated by α_2 on the abscissa in Fig. 2. The two methods of altering the simulated data (see above) yielded boundaries around the resolved components within which all actual measurements would be expected to fall. Thus, if a real DPH/membrane sample were measured, which contained 10- and 5-ns components in a 4:1 intensity ratio ($\alpha_2 = 0.2$), we could reasonably expect to resolve τ_1 within the range 9.9–10.2 ns, τ_2 within the range 4.2–5.8 ns, and α_2 within the range 0.15–0.27 (see inset to Fig. 2). For the cases in Fig. 2, reasonable accuracy of resolution is obtained down to a value approaching 0.05 for α_2. Thus, if the lifetimes of a two-component sample are 10 and 5 ns, we expect to be able to resolve them even if the fluorescence intensity of one component is only on the order of 5% of the total.

To test the generality of this result for a wide range of

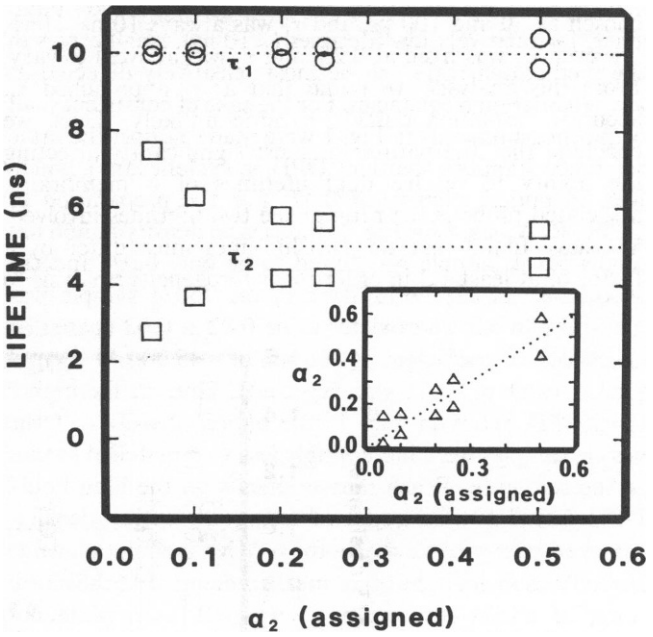

FIGURE 2 Effect of probable errors in phase and modulation lifetimes (see the section Precision of DPH Lifetime Measurements in Membranes) on two-component resolution as a function of the fraction of secondary component. Resolved lifetime components (principal lifetime, τ_1, circles; secondary lifetime, τ_2, squares; fraction of secondary component, α_2, triangles in *inset*) are plotted vs. the assigned fraction of secondary component used to generate hypothetical data (according to Eq. I.29 of reference 14). Hypothetical observed phase and modulation lifetimes were altered by small errors (50–100 ps), as described in Materials and Methods. Lifetime and secondary component values assumed in generating the hypothetical data are indicated by dotted lines. The resolved components delimit the range of possible errors in two-component lifetime heterogeneity analysis with the current level of precision (see text). For each of the parameters τ_1, τ_2, and α_2, the higher values were obtained from increasing the phase and decreasing the modulation lifetimes, while the lower values resulted from the opposite procedures (see text).

[3] F_{12} is the ratio of the reduced chi-square values (X^2_ν = X^2/degrees of freedom) of the single component relative to the two component fit. This ratio is a measure of the improvement of the two-component fit compared with the single-component fit, such that smaller values indicate greater probability that a single-decay model provides an adequate description of the data (27). The value of F_{12} determines the probability, P, that the improvement in reduced chi-square for the two-component fit is due merely to statistical errors in the data (27). We have taken a value of $P >$ 0.5 as an indication that the data were adequately described by a single-exponential model.

lifetimes, we performed another set of hypothetical resolutions, and the results are presented in Fig. 3. Here the ratio of τ_1/τ_2 was maintained at 2.0, and the relative intensities were fixed at $\alpha_2 = 0.25$ for all cases, while the assigned value of τ_1 was varied from 2 to 100 ns. Error margins used for this simulation were equivalent to the phase angle or modulation ratio errors corresponding to the probable errors of 50–100 ps described above. This procedure properly weights the probable lifetime errors established for a 10-ns sample for application to considerably different lifetimes (i.e., <5 ns and >20 ns). The results depicted in Fig. 3 indicate that the most difficult region for lifetime resolution lies below $\tau_1 = 5$ ns. For longer lifetimes, accurate two-component resolution should be possible. Thus, the range of average DPH lifetimes in membranes (7–12 ns) is well suited to examination with the phase fluorometer (SLM 4800; SLM Instruments).

Finally, one more set of simulations was performed to test the reliability of lifetime heterogeneity analysis as a function of the relative magnitudes of τ_1 and τ_2. The results are presented in Fig. 4. As in Fig. 2, the error margins were chosen as 50 and 100 ps, and τ_1 was always 10 ns. Here, however, α_2 was fixed at 0.25, and τ_2 was allowed to vary. From this analysis, we found that as τ_2 approached τ_1, accurate resolution quickly became unlikely. Thus, we conclude that the parameter most significantly affecting the ability to resolve dual lifetimes of a membrane-associated probe is the ratio of the two lifetimes involved. We have found that the two lifetimes must differ by a factor of at least 1.3 in order for heterogeneity analysis to

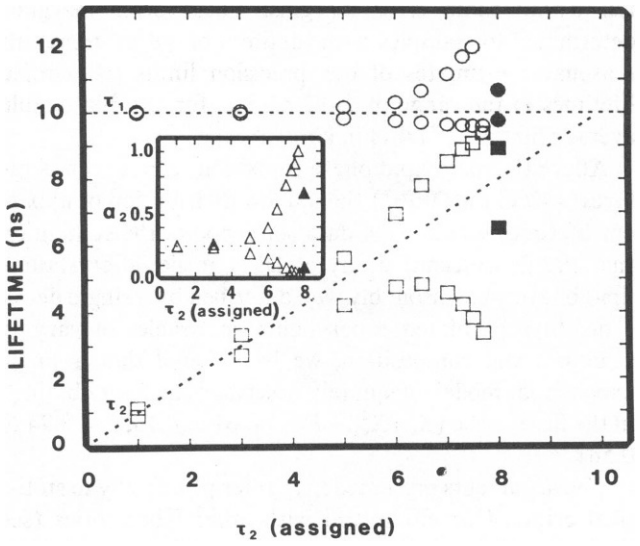

FIGURE 4 Effect of probable errors in phase and modulation lifetimes on two-component lifetime resolution as a function of the relative magnitudes of the component lifetimes. The symbols are as in Fig. 2. For a fixed fraction of secondary component ($\alpha_2 = 0.25$), this figure shows the maximal error of resolved component lifetimes as a function of the magnitude of the secondary lifetime, relative to a fixed (10 ns) primary lifetime. As in Fig. 2, larger resolved lifetimes resulted from increasing the phase and decreasing the modulation lifetimes (see text). The filled symbols (at τ_2 [assigned] = 8 ns) represent calculated maximal analysis errors obtained with probable observed lifetime errors reduced by a factor of four (see text). Assumed values of τ_1, τ_2, and α_2 (*inset*) are shown by dotted lines.

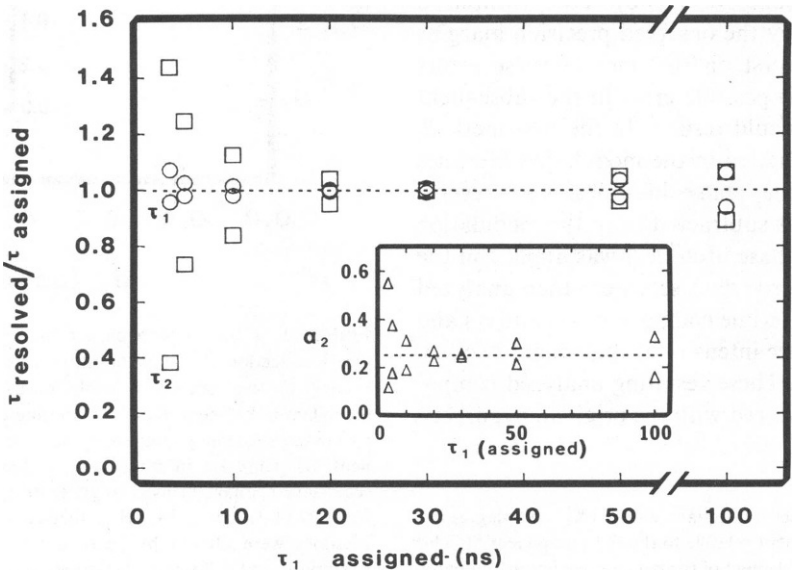

FIGURE 3 Effect of probable errors in phase and modulation lifetimes on two-component lifetime resolution as a function of the magnitude of the component lifetimes. The results of simulations are shown as the ratio of the resolved divided by the assigned lifetimes, with the same symbols as in Fig. 2, including dotted lines indicating the correct (assigned) values. In generating hypothetical phase and modulation lifetimes by Eq 1 and 2, τ_1/τ_2 was fixed at 2.0, and α_2 (*inset*) was assigned to be 0.25. Because of the broad range of lifetimes considered here, the errors introduced were errors in phase angle or modulation ratio equivalent to the 50–100 ps lifetime errors characteristic of 10-ns lifetime samples (see text). For all cases in which the assigned τ_1 was <30 ns, the higher set of resolved values resulted from increasing the phase and decreasing the modulation lifetimes in the hypothetical data sets (see text). Also note the break in the abscissa for τ_1 between 50 and 100 ns.

be reliable. Note that this limitation has been derived for $\alpha_2 = 0.25$. The limiting ratio of 1.3 is roughly valid for α_2 down to 0.2, although the value of 1.3 is expected to increase gradually as α_2 decreases, as suggested by Fig. 2.

Our analysis has been in terms of worst possible errors generated as described above. It is instructive to consider instead the probable error in the resolved lifetime components rigorously derived from the assumed errors in the apparent phase and modulation lifetimes at different frequencies. This was done using the expression (27)

$$\sigma_x^2 \simeq \sum_i \sigma_i^2 \frac{(\delta x)^2}{(\delta \tau_i)},$$

where σ_x^2 is the variance in a parameter x (i.e., τ_1, τ_2, or α_2) and τ_1 represents the apparent lifetimes. The expected errors (σ_x) for the case $\tau_2 = 7.5$ ns, $\tau_1 = 10$ ns, and $\alpha_2 = 0.25$ (see Fig. 4), as determined in this way, were ± 1.1 ns for τ_1, $+4.9$ ns for τ_2 and ± 0.4 for α_2. These errors are quite similar to the maximal errors recorded in Fig. 4, and justify our use of the more easily calculated maximal errors.

Experimental Tests of Lifetime Heterogeneity Analysis with DPH-labeled Vesicles and Quinine Sulfate

We have recently detailed conditions under which the commonly used fluorophore quinine sulfate exhibits nearly single-lifetime behavior (16). The quinine fluorescence decay can be conveniently adjusted to lower lifetimes (unquenched value of 19.4 ns) by the presence of chloride ion (roughly 0.24 M NaCl reduced the quinine lifetime to 4.4 ns), but still remains reasonably well approximated by a single fluorescence lifetime. In Table I, we present results for the fluorescence heterogeneity analysis of mixtures of DPH-labeled vesicles with solutions of quinine sulfate containing varying concentrations of NaCl.

Comparison of the resolved and expected components in Table I illustrates several of the conclusions of the previous theoretical simulations. First, the primary fluorescence lifetime, τ_1, is generally determined more accurately than is τ_2. Second, the accuracy of the resolution is generally greater in the mixtures where τ_1/τ_2 is roughly 2.0 than in those where τ_1/τ_2 approaches 1.3. Third, for all samples in Table I, the heterogeneity analysis has resolved the component lifetimes with at least reasonable accuracy (compare with the error ranges in Fig. 4). This reinforces our assertion that errors in the raw lifetime measurements are within the 50–100 ps range that we have routinely used for heterogeneity analyses.

TABLE II
AVERAGE DPH LIFETIMES IN DIFFERENT DPPC VESICLE PREPARATIONS

Vesicle	Average lifetime	
	above T_m (49–50°C)	below T_m (32–39°C)
	ns	*ns*
DOVs	—	12.58
REVs - X*	8.12	10.79
REVs - 0.4‡	8.41	11.44
REVs - 0.2§	8.61	11.67
FUVs	—	10.92
SUVs	8.25	9.89
LMVs	7.85	9.56
LMVs - 0.4‡	—	10.98

The average lifetime, $\alpha_1\tau_1 + \alpha_2\tau_2$; T_m, main phase transition temperature (~41°C in DPPC LMVs); DOVs, unilamellar vesicles prepared by slow dialysis of octylglucoside suspensions (22); FUVs, unilamellar vesicles prepared by fusion of SUVs (23).
*Not extruded through any polycarbonate filters (see Methods).
‡Extruded through 0.4 μm filter.
§Extruded through 0.4 and 0.2 μm filters.

DPH Lifetime Behavior in Different Lipid Vesicle Types

Before we present our results on the resolution of multiple DPH lifetimes in lipid bilayers, we must first address a

TABLE I
RESOLUTION OF FLUORESCENCE LIFETIMES FROM MIXTURES OF QUININE SULFATE AND DPH-LABELED VESICLES

τ_1		τ_2		α_2		Expected
Resolved	Expected	Resolved	Expected	Resolved	Expected	τ_1/τ_2
19.29	19.40	7.85	8.98	0.12	0.19	2.16
11.71	12.37	6.74	8.98	0.12	0.41	1.38
12.78	12.37	9.04	8.98	0.31	0.26	1.38
8.71	8.98	4.49	4.40	0.48	0.48	2.04

Resolved lifetime components at 23°C from mixtures of aqueous, partially quenched quinine sulfate and DPH in SR lipid REVs. The aqueous medium contained 0.1N H_2SO_4, with varying amounts of NaCl added to quench the quinine lifetime (16). Errors of 0.05 ns (for 6-MHz phase, 18-MHz phase and modulation, 30-MHz modulation) and 0.1 ns (6-MHz modulation, 30-MHz phase) were used for the lifetime heterogeneity analysis (see Materials and Methods). The expected lifetimes τ_1 (lifetime of the component with greatest fluorescence intensity) and τ_2 (lifetime of the second component) were determined by measuring the quinine sulfate and DPH-labeled vesicle lifetimes individually before mixing. The expected fractional fluorescence intensity of the second component, α_2 (equal to $1 - \alpha_1$), was determined from steady-state fluorescence intensity measurements of the individual samples and the known volumes used in the mixtures. Expected τ_1/τ_2 is the ratio of the two known lifetime components. Two samples with expected $\tau_1/\tau_2 = 1.38$ are shown. The analysis of these illustrate the uncertainty in resolved components that can be expected when resolving two closely spaced lifetimes.

rather surprising observation. We have found, particularly in the solidlike phases (gel phases) below the main lipid phase transition, that the average DPH lifetime differs significantly between different types of vesicles (Table II). In general, the largest average DPH lifetimes were observed in intermediate-sized, unilamellar vesicles. While all the data shown in Table II are for DPPC vesicles, we have observed similar results for DMPC and 1,2,-dipentadecanoyl-3-sn-phosphatidylcholine. While there may be reason to suspect that the intermediate-size REVs and dialyzed octylglucoside vesicles (which show the largest DPH lifetimes) are affected by residual impurities from the solvent or detergent with which they were prepared (20) it is interesting to note that extrusion of LMVs through a (presumably inert) polycarbonate filter also leads to a longer DPH average lifetime, as shown at the bottom of Table II. Fused, unilamellar vesicles, which were prepared from SUVs without solvents or detergents (23) and appear (by high-sensitivity, differential scanning calorimetry) to be free of impurities (20), also had a longer average lifetime than LMVs or SUVs (Table II).

While the relationship between vesicle type and DPH average lifetime is most clearly seen in gel-phase lipids, a qualitatively similar but less distinct pattern has been observed above the main phase transition temperature, as is also shown in Table II. The highest average DPH lifetimes were observed in unilamellar vesicles of roughly 1,000 Å diameter, with smaller average lifetimes seen in SUVs (250 Å diameter) and LMVs (>2,000 Å average diameter). Although several factors may contribute to the fluorescent lifetime of DPH in a bilayer, to a first approximation, the lifetime of DPH probably correlates with lipid packing density. In this light, the data in Table II would seem to imply that the lipid packing density is different in intermediate-size unilamellar vesicles, as compared with extremely small, unilamellar or large, multilamellar vesicles.

Temperature Dependence of DPH Lifetime

To more completely document the lifetime behavior of DPH in lipid vesicles, Fig. 5 shows the temperature dependence of the observed phase and modulation fluorescence lifetimes and the resolved lifetime components of DPH in DPPC LMVs. Above the main lipid phase transition (41.3°C), only one DPH lifetime component was resolved, as described above. Below the main transition, however, DPH lifetime heterogeneity was found at all temperatures. While the main ($P'_\beta \leftrightarrow L_\alpha$) transition of the lipids was clearly visible, both in the observed (Fig. 5 *A*) and the resolved (Fig. 5 *C*) lifetime data, the pretransition ($L'_\beta \leftrightarrow P'_\beta$, ~30°C, [28]) could not be detected. An interesting feature of the data in Fig. 5 *C* was the decrease of the average DPH lifetime ($\langle\tau\rangle = \alpha_1\tau_1 + \alpha_2\tau_2$) with decreasing temperature below the main transition, apparently caused by an increase in the resolved contribution of the secondary lifetime component (α_2).

Although we have found substantial differences in the average DPH lifetime for different types of DPPC vesicles (Table II), the pattern of lifetime heterogeneity in SUVs and REVs (not shown) as a function of temperature was nearly identical to that observed for LMVs (see Fig. 5). The only significant dissimilarity was the broadened temperature range of the main phase transition in SUVs, which agrees with the well-documented phase behavior of these vesicles (29). Otherwise, the decrease of the average DPH lifetime with decreasing temperature, which can be seen in Fig. 5 *C* for LMVs, was also evident in SUVs and REVs. We note that this decrease can also be seen (but was not noted) in the data of Kawato et al. (Fig. 6 of reference 3), for DPH in sonicated DPPC liposomes. While the lifetime of DPH in most isotropic solvents decreases monotonically or remains constant with increasing temperature (1, 30, 31), it has been observed to increase on heating from very low temperature (−100 to 0°C) in 3-methyl-pentane or methylcyclohexane (30). This may reflect a similarity between the ordered environment experienced in a gel-phase bilayer and the local environment in these organic solvents at very low temperature.

In addition, below the main phase transition, we observed DPH lifetime heterogeneity in LMVs (Fig. 5), SUVs (not shown), and REVs (see below) that could be well fitted in terms of a long lifetime (~10–12 ns) component and a small amount (~5–20%) of a short lifetime (~3–8 ns) component. This disagrees with Klausner et al. (7), who analyzed their phase and modulation data in terms of a single DPH lifetime in DPPC liposomes at 25°C. Note, however, that the reported uncertainty in their measured lifetimes (7, their Table VI) is roughly ten times greater than the precision of our measurements, and could account for their inability to detect the amount of heterogeneity that we have resolved.

Although interpretation of the secondary DPH lifetime component (τ_2) in gel phase lipids is far from unambiguous, the low values (see Fig. 5 *C*) observed for this parameter seem to suggest the existence of regions of disrupted lipid packing where the probability of water penetration would be greater. Such regions have already been proposed to exist at the interface of lateral domains in lipid mixtures (9), and at the protein-lipid interface in protein-containing membranes (17).

Experimental Tests of DPH Lifetime Heterogeneity Analysis with Well-defined Membrane Systems

To directly test our ability to accurately resolve dual DPH lifetime components, membranes were needed in which DPH could be expected to display, at least to a good approximation, single exponential fluorescence decay. If two such membranes having significantly different DPH lifetimes could be found, then a mixture of the two would provide an ideal test system for heterogeneity resolution.

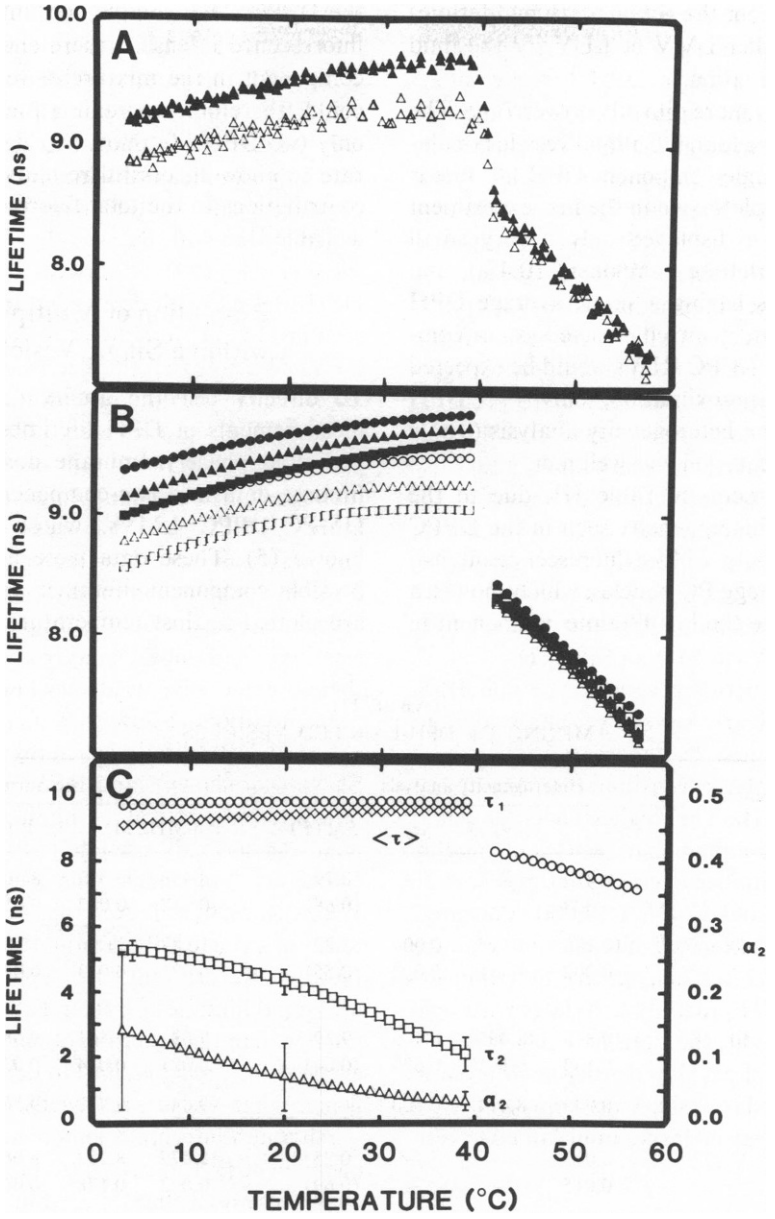

FIGURE 5 Temperature dependence of the fluorescence lifetime behavior of DPH (1 per 1,000 lipids) in DPPC LMVs dispersed in 50 mM KCl. (*A*) Observed lifetimes from modulation (filled triangles) and phase (open triangles) measurements at a modulation frequency of 18 MHz. The data from four cooling scans (-45°C h^{-1}) are superimposed, two each at lipid concentrations of 0.1 and 0.2 mM. (*B*) Least-squares quadratic fits to the observed lifetime data were obtained separately for the temperature regions (3–39 and 42–56°C) below and above the main DPPC phase transition (41.3°C). Data smoothed by this fitting procedure are shown for phase shift (open symbols) and modulation ratio (filled symbols) measurements at modulation frequencies of 6 MHz (circles), 18 MHz (triangles, original data plotted in Fig. 5 *A*), and 30 MHz (squares). (*C*) Resolved DPH lifetime components obtained from heterogeneity analysis of the fitted phase and modulation data shown in *B*. The principal lifetime component, τ_1, is depicted by circles, the secondary lifetime component, τ_2, is represented by squares, and the average lifetime ($\langle\tau\rangle = \alpha_1\tau_1 + \alpha_2\tau_2$) is shown by diamonds. The fractional fluorescent intensity, α_2, is represented by triangles and plotted with respect to the right ordinate. Above the main DPPC phase transition, only a single DPH fluorescence lifetime was required to adequately fit the data. Substantial uncertainty in τ_2 and especially α_2 was indicated by the heterogeneity analysis procedure and is shown by error bars in the figure.

There were two major problems in designing these experiments. First, the difference between DPH lifetimes in the gel and fluid phases of any single vesicle type was insufficient (i.e., their ratio was <1.3) to allow resolution. Second, no gel phase vesicle could be found in which DPH displayed only a single component (homogeneous) fluorescence decay. The first problem was circumvented by using two different types of vesicles. As noted above, the average DPH lifetime in LMVs was found to be somewhat smaller than the lifetime in REVs and LUVs, especially in the gel phase, as shown in Table II. Thus, the difference between the lifetime of DPH in gel and fluid phases can be

maximized by using LUV for the gel phase (long lifetime) vesicle population and either LMV or LUV for the fluid phase (short lifetime) population.

The second problem was more difficult to overcome. The best approximation we have found to a lipid vesicle population displaying a large, single-component DPH lifetime is the 40% Chol/DPPC sample shown in the first experiment of Table III. This sample displayed only a very small percentage of a second lifetime component (0.4%), and could be approximated as having a single average DPH lifetime of 10.94 ns. Thus, a mixed vesicle system composed of these vesicles and DPPC REVs would be expected to display, to a good approximation, only two DPH lifetimes. The results of the heterogeneity analysis (Table III) indicate that this expectation was well met.

For the second experiment in Table III, due to the significant DPH lifetime heterogeneity seen in the DPPC sample, we chose to mix only ~14% (fluorescence intensity) of this sample with the egg PC vesicles, which showed a single DPH lifetime. Since the low-lifetime component in the DPPC REV sample accounted for ~20% of the total fluorescence intensity, there should be only ~3% of this component in the mixture of vesicle populations, and we could, therefore, approximate the mixture as displaying only two DPH lifetimes. Our data were sufficiently accurate to allow successful resolution of the expected major contributions to the total fluorescence decay, as indicated in Table III.

Resolution of Multiple Bilayer Domains within a Single Vesicle Population

To directly test the ability of phase and modulation measurements of DPH lifetimes to detect coexisting gel and fluid phase membrane domains, we have recorded lifetime data in a two-component membrane system, 1:1 DMPC/DPPC LMVs, whose phase behavior is well known (5). These data were analyzed in terms of two possible component lifetimes, and the resultant lifetimes are plotted against temperature in Fig. 6. In this figure,

TABLE III
MIXING OF DPH-LABELED VESICLES

Experiment	Sample	Temperature	Heterogeneity analysis				Measured lifetimes					
			τ_1	τ_2	α_2	F_{12}; (P)	P 6 MHz M		P 18 MHz M		P 30 MHz M	
I	1. DPPC REVs	50	8.414	—	—	0.79	8.438	8.312	8.400	8.400	8.392	8.453
			+/− 0.003	—	—	(0.68)	+/−0.017	0.083	0.011	0.027	0.024	0.015
	2. 40% CHOL/DPPC REVs	50	10.980	1.117	0.004	1.27	10.88	11.06	10.88	10.99	10.84	10.96
			+/− 0.002	1.431	0.002	(0.55)	+/−0.021	0.033	0.074	0.030	0.043	0.015
	Mixture: 1:1	50	10.813	8.465	0.455	9.39	9.681	9.861	9.580	9.730	9.517	9.685
			+/− 0.133	0.171	0.609	(0.04)	+/−0.021	0.034	0.030	0.051	0.043	0.015
	Expected		10.943	8.414	0.500	—	9.640	9.772	9.500	9.657	9.428	9.585
II	1. Egg PC REVs	39	8.092	—	—	0.75	8.077	8.123	8.069	8.128	8.136	8.078
			+/− 0.015	—	—	(0.69)	+/−0.092	0.178	0.061	0.137	0.123	0.056
	2. DPPC REVs	39	11.870	7.930	0.214	43.9	10.96	11.23	10.72	10.98	10.52	10.91
			+/− 0.111	0.404	0.421	(0.005)	+/−0.059	0.142	0.102	0.059	0.126	0.118
	Mixture: 6:1	39	7.960	12.66	0.114	11.7	8.429	8.659	8.275	8.484	8.301	8.379
			+/− 0.094	0.721	0.345	(0.03)	+/−0.065	0.195	0.059	0.060	0.131	0.081
	Expected		8.092	11.03	0.138	—	8.473	8.588	8.359	8.490	8.359	8.444

Measured and resolved (by heterogeneity analysis) lifetimes of DPH in lipid vesicles. In each experiment, measurements were made on two individual vesicle populations, which were then mixed together and reexamined. The analysis is presented in terms of three parameters (unless the sample was found to have only a single lifetime): τ_1 (lifetime of the component with greatest fluorescence intensity), τ_2 (lifetime of the second component), and α_2 (fractional fluorescence intensity of the second component, equal to $1 - \alpha_1$). F_{12} and P (in parentheses) are statistical parameters used to compare the one- and two-component fits (see footnote 3). The standard deviations (indicated by +/−) are the actual standard deviations of 10 measurements (each of which was the instrumental average of 10 signals) acquired in ~30 s for both phase (P) and modulation (M) at a given frequency with the fluorometer (4800 SLM; SLM Instruments) (12). The unexpectedly small errors associated with the lifetime of the single component systems (I-1 and II-2) reflect statistical reduction of the estimated parent population variance resulting from averaging six independent, nearly equal measurements (reference 27, section 5.1). The expected fractional intensity (α) was determined from steady-state fluorescence intensity measurements of the individual samples and the known volumes used in the mixture. This value for the expected α agreed closely with the value obtained from combining the average lifetimes of the mixed and individual samples, using $\alpha_1 = (\langle\tau\rangle - \tau_2)/(\tau_1 - \tau_2)$. The expected measured lifetimes were generated from the known lifetimes and proportions of the individual components using eqs. I.29 of reference 14. The expected lifetimes, τ_1 and τ_2 are simply the values measured for the individual vesicle preparations, with a few adjustments necessitated by the lifetime heterogeneity of some of the individual samples. For example, in experiment I, the expected τ_1 (10.94 ns) was the average lifetime ($\langle\tau\rangle = \alpha_1\tau_1 + \alpha_2\tau_2$) of the 40% Chol/DPPC REVs. In experiment II, the expected τ_2 (11.03 ns) was the average lifetime of the DPPC REVs at 39°C.

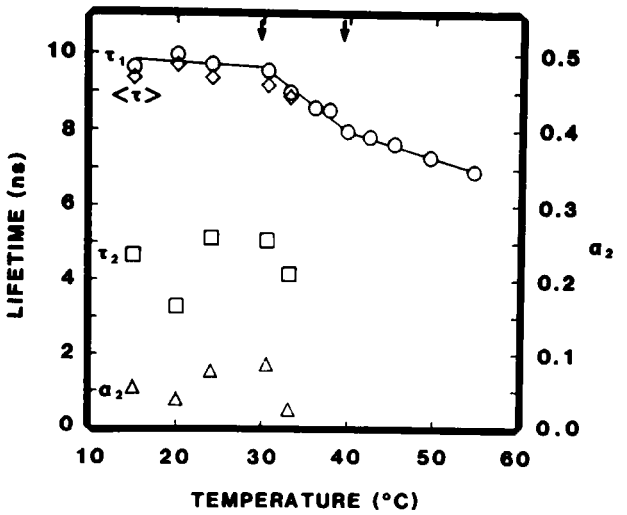

FIGURE 6 Temperature dependence of resolved DPH lifetimes in 0.2 mM DPPC/DMPC (1:1) LMVs prepared in 50 mM KCl. Arrows at the top of the figure indicate previously determined (5) phase boundary temperatures for the main transition region in this system. Above 35°C, only a single lifetime component was required to fit the data, while lifetime heterogeneity was observed at lower temperatures. τ_1, τ_2, $\langle\tau\rangle$, and α_2 are indicated by circles, squares, diamonds, and triangles, respectively, as in Fig. 5 *C*. Three, straight-line segments have been included to point out the breaks in τ_1 at the phase boundaries.

arrows denote the temperatures of thermodynamic phase boundaries, which have been determined previously by steady-state DPH fluorescence measurements (5).

In the mixed DPPC/DMPC system, a single DPH lifetime was observed in the high temperature (L_α phase) region (see Fig. 6), just as we had found for each of the individual lipid components. We continued to observe a single DPH lifetime below 41°C (the main transition temperature for pure DPPC LMVs), consistent with the previous observation that the liquid-crystalline (L_α) phase exists down to 39°C in 1:1 DMPC/DPPC LMVs (5). While the average (single component) lifetime data in Fig. 6 did show an inflection at ~39°C which corresponds with the onset of the phase separation observed in this system (5), we were unable to resolve DPH lifetime heterogeneity until the sample temperature dropped below 35°C (see Fig. 6). However, the two lifetimes resolved below 35°C were similar to those seen in the gel phase of pure DPPC (~10 and 4.5 ns, see Fig. 5 *C*) rather than those expected for coexisting fluid and gel phases (10 and 8 ns). The principal (τ_1) and average DPH lifetimes became constant at 30°C, which correlates with the disappearance of fluid phase lipid inferred from the phase diagram for this mixed lipid system.

Thus, DPH lifetime heterogeneity in mixed DMPC/DPPC vesicles appears to reflect only the presence of gel-phase lipid, rather than the coexistence of gel and liquid crystalline phases. This probably reflects the coexistence of three DPH lifetimes in the two-phase DMPC/DPPC vesicles: ~8 ns from the fluid domains and both 10 and 4–5 ns components from the gel-phase domains. When the data were analyzed in terms of only two components, the 8- and 10-ns components apparently were resolved as a single component that varied from ~8.0 to 9.8 ns between 39 and 30°C (see Fig. 6). Once present at a level $\gtrsim$2% of the total fluorescence intensity, the 4–5-ns component appears to have been sufficiently different from the two larger components to be resolved by our heterogeneity analysis.

To test the interpretation of Fig. 6 outlined above, we assumed that the gel phase in these vesicles could be characterized by a DPH lifetime (τ_G) of 9.5 ns ($\langle\tau\rangle$ below 30°C), and that the average DPH lifetime in the fluid phase (τ_F) varied linearly from 8.1 ns at 39°C to 8.5 ns at 31°C (values obtained by extrapolation of the average lifetime data from the region above 40°C). With these assumptions, the fractional contribution of fluorescence from DPH in the gel phase (α_G) could be obtained as $\alpha_G = (\langle\tau\rangle - \tau_F)/(\tau_G - \tau_F)$. The agreement between the results of this analysis and the relative amounts of gel and fluid phases determined from the steady-state fluorescence-derived phase diagram for this system (5) was excellent (correlation coefficient = 0.993; data not shown). Thus, for temperatures at which gel and fluid phases coexist, there appear to be at least three contributions to the observed DPH fluorescence decay, one (~8 ns) from fluid phase regions and two (4–5 ns and 9.5 ns) from gel-phase regions. However, our measurements could resolve only two lifetime contributions, such that the major DPH lifetime component in mixed phase vesicles reflected the average of the 8-ns and 9.5-ns contributions.

Note that these results are at variance with previous reports that gel- and fluid-phase domains within the same membrane can be resolved in terms of DPH lifetimes characteristic of these two microenvironments (7–9). Although the precision of our data and our methods for eliminating systematic errors represent the best so far reported for membrane samples with commercial phase and modulation fluorescence instrumentation, we have been unable to reproduce experimentally the resolution of DPH lifetimes in coexisting membrane domains as described by Klausner et al. (7). In addition, our analysis of probable errors (see above) indicates that such an experiment would be at the limits of feasibility even under the best conditions, using the commercially available instrument.

Controversy over DPH Lifetime Heterogeneity in Fluid Phase Membranes

One inescapable conclusion from our data is that DPH fluorescence decay in the fluid phase of model, phospholipid membranes is reasonably well described by a single exponential function, but displays multi-exponential decay in the gel phase. This conclusion, however, is a controversial one. Several workers have addressed this issue using either pulse or phase shift methods and have either pub-

lished (3, 7, 32, 33) or relayed to us their results (A. G. Szabo; S. Georghiou; E. Gratton, personal communications). Not only do the results from different laboratories disagree quantitatively, but there is no general concensus on the qualitative issue of whether DPH fluorescence displays a single- or double-exponential decay from either the fluid or gel phase. Indeed, every possible combination of qualitative results has been obtained. Given the several potential artifacts that we have found associated with making phase and modulation measurements on membrane suspensions, the confusion on this point is not surprising. Very recently, an additional artifact has been unearthed through the cooperation of our laboratory with the laboratories of Drs. E. Gratton and A. G. Szabo, who made lifetime measurements on our DPH-containing vesicles using some of the most current and sophisticated phase/modulation or pulse techniques, respectively. In all three laboratories, DPH in both DPPC LMVs and REVs carefully protected from light showed nearly single exponential fluorescence decay (fractional intensity of second component = $\alpha_2 \leqq 0.03$ to 0.05) above the phospholipid phase transition. This is at the limit of our ability to resolve the 2 to 3-ns component with the phase fluorometer (SLM 4800; SLM Instruments). However, samples exposed to normal laboratory light levels for only 3 to 6 h displayed dual exponential decay with a minor 2 to 3-ns lifetime component having a fractional intensity of 0.05 to 0.08. This component was clearly resolved in all three laboratories. This suggests that a photodecomposition product of either lipid or DPH is responsible for the 2 to 3-ns minor lifetime component often reported for DPH-containing, fluid-phase membranes (32, 33, 36). Gratton's group has come to the same conclusion on the basis of studies stimulated by the exchange of samples between our laboratories (37). However, Gratton's group (37) also reported that DPPC vesicles exposed to light before addition of DPH showed appreciable 2 to 3-ns component, while, in our hands, no 2 to 3-ns component was observed under these conditions. It is safe to say that the 2 to 3-ns component associated with DPH fluorescence decay is due to a photodecomposition reaction, but the exact origin of this component remains to be more fully explored.

SUMMARY

Given the level of precision achieved here for phase and modulation measurements of DPH lifetimes in membranes, we have evaluated theoretically the limits of accurate heterogeneity resolution in hypothetical, two-component systems. The results obtained suggest that if the two lifetime components differ by a factor of 1.3 or more, then they can be accurately resolved, at least for lifetimes in the range of 5–100 ns (see Figs. 2–4). We note, however, that it may not be possible to achieve such small errors for all phase and modulation measurements over this large a range of lifetimes, due to the nonlinear character of the phase and modulation response functions (13).

Within the range of average DPH lifetimes normally observed in membranes (~7–12 ns), if the ratio of the coexisting lifetimes drops below ~1.3, the level of precision currently possible would not assure accurate resolution. The procedures used in generating Fig. 4 have revealed that improvement of the experimental precision by a factor of four would not substantially improve our ability to resolve multiple lifetimes. Interestingly, a similar limitation (τ_1/τ_2 must exceed 1.2 for two-component resolution) has recently been reported as a theoretical limit for pulse fluorescence decay measurements (34).

It has already been suggested that three-component analysis (where all three components are of the same order of magnitude) cannot be uniquely performed using commercial phase fluorometers even with the level of precision achieved here (9), unless additional spectral information is available (35). To our knowledge, data of greater precision have not been obtained on currently available commercial instrumentation. Thus, efforts to experimentally resolve dual lifetimes in DPH-labeled membranes can be expected to be successful only if the following two criteria are met. (*a*) The two populations of DPH in vesicles must have lifetimes differing by at least a factor of 1.3, and (*b*) the two-population sample must demonstrate, as closely as possible, only two lifetime components, because the presence of an appreciable third component would hinder accurate two-component resolution.

For the two examples shown (see Table III) in which successful resolution of well-defined, mixed-vesicle populations was achieved, the ratios of the lifetimes of the components were 1.3 and 1.5, thus meeting the first criterion. The second criterion (only two components in the mixture) was approximately satisfied in the experiments of Table III by adjusting the amount of any third lifetime component to a very low level. The magnitude of the errors in these mixture experiments supports the prediction made on the basis of our calculations (e.g., Fig. 4) that these mixtures of carefully chosen vesicle systems approach the current limits of accurate heterogeneity resolution.

While there may be significant limitations on the accuracy with which heterogeneous emissions may be resolved, the phase and modulation technique now appears to be highly accurate for the determination of single-component fluorescence lifetimes in membrane systems, being limited mainly by the accuracy of the lifetime assignment of reference standards (12). Also, the average lifetime ($\langle\tau\rangle$) of multiple-lifetime systems can be determined with similar accuracy (12). Thus, differences between various types of DPPC vesicles (see Table II) can be sensitively detected by determination of average DPH lifetimes. Contamination of an essentially homogeneous population can be detected (even if it cannot be accurately resolved) at levels approaching 5%, if the contaminant differs in lifetime from the main component by a factor of 2 (see Fig. 2). This can be conveniently accomplished by comparing the reduced chi-square parameter for one and two-component fits on

the same data set (12; see footnote 3; see also legend to Table III of this work).

Despite the accuracy that we have achieved with phase and modulation techniques as applied to membrane systems, we must conclude that coexisting gel and fluid phase membrane domains are not likely to be resolved using currently available commercial instrumentation except in those special instances when the lifetimes of DPH in the two domains are sufficiently distinct. At least from this standpoint, a different fluorescent probe which has a greater lifetime difference in gel and liquid-crystalline lipid phases (e.g., parinaric acid) might be more useful in resolving coexisting membrane domains. Also, spectral differences associated with different probe domains (not applicable to DPH) could be exploited for the improved resolution recently established for global analysis of fluorescence heterogeneity (34, 35). A further complication, however, is that a membrane with coexisting lipid domains is likely to contain an interfacial region (with disrupted lipid packing) between the different domains (7). Thus, there could exist three different probe environments (and therefore three lifetime components), and accurate resolution would be extremely difficult. Certainly, however, the potential biological significance of membrane domains makes efforts to perfect this approach worthwhile. Improved instrumentation as well as characterization of other fluorescent probes could contribute significantly to solving the problems that we have defined above. In this regard, Parasassi et al (37) reported, while our manuscript was in review, the successful resolution of fluorescence lifetime contributions from DPH in coexisting gel (9.5 ns) and fluid (7.5 ns) membrane domains ($\tau_1/\tau_2 = 1.27$) using a newly developed multifrequency phase fluorometer (26). Despite the fact that Parasassi's de novo analysis was obscured by a short lifetime component, as was ours (see discussion of Fig. 6), the additional precision available with this new instrumentation offers great promise for the resolution of membrane domains through lifetime analysis.

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