RESOLVABILITY OF FLUORESCENCE LIFETIME DISTRIBUTIONS USING PHASE FLUOROMETRY

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ABSTRACT The analysis of the fluorescence decay using discrete exponential components assumes that a small number of species is present. In the absence of a definite kinetic model or when a large number of species is present, the exponential analysis underestimates the uncertainty of the recovered lifetime values. A different approach to determine the lifetime of a population of molecules is the use of probability density functions and lifetime distributions. Fluorescence decay data from continuous distributions of exponentially decaying components were generated. Different magnitudes of error were added to the data to simulate experimental conditions. The resolvability of the distributional model was studied by fitting the simulated data to one and two exponentials. The maximum width of symmetric distributions (uniform, gaussian, and lorentzian), which cannot be distinguished from single and double exponential fits for statistical errors of 1 and 0.1%, were determined. The width limits are determined by the statistical error of the data. It is also shown that, in the frequency domain, the discrete exponential analysis does not uniformly weights all the components of a distribution. This systematic error is less important when probability and distribution functions are used to recover the decay. Finally, it is shown that real lifetime distributions can be proved using multimodal probability density functions. In the companion paper that follows we propose a physical approach, which provides lifetime distribution functions for the tryptophan decay in proteins. In the third companion paper (Alcala, J. R., E. Gratton, and F. J. Prendergast, 1987, *Biophys. J.*, in press) we use the distribution functions obtained to fit data from the fluorescence decay of single tryptophan proteins.

INTRODUCTION

The fluorescence lifetime of a simple molecule is determined by several factors, including the presence of neighboring molecules, the dielectric constant of the medium, temperature, and pressure. For a simple molecule in a dilute gas, the probability of emission can be calculated from physical principles. When only one mechanism for deactivation of the excited state is present, the fluorescence decays exponentially. In the condensed phase different decay laws can arise. In a high pressure gas the rate of decay can be determined by the rate of collisions. In a viscous medium, the presence of "stable" (during the excited state lifetime) microenvironments can cause a deviation from the exponential law. In systems that exhibit energy transfer, the distribution of acceptor molecules can determine the law describing the decay (1). Given the variety of processes that can generate a deviation from exponentiality, it is rather surprising to find fluorescent systems in which the decay can be well approximated by a single exponential. In most of the biological systems, proteins, membranes, and nucleic acids, the conditions that will give rise to a nonexponential decay should prevail. In fact in most of these systems the fluorescence decay is usually described using more than one exponential. The physical interpretation being that the number and relative contribution of the exponential components can be related to the number and quantity of the different species present in the system under examination. It is important to examine under which conditions this interpretation can be valid and if a double exponential decay reflects the existence of two species or rather is an approximation of the decay curve due to the limited accuracy of the experimental apparatus used. A simple rule is that if only one decade in time, or frequency, is collected, it is rather difficult to prove the nonexponential character of the decay unless the precision of the measurement is very high. Data collected over two decades can be used to detect two exponentials and at least three decades are needed for three exponentials. In this respect, a striking difference exists between data collected in the time and in the frequency domains. In the time domain, it is customary to collect approximately 1,000 data points equally spaced in the time axis and the uncertainty of each point varies from 0.5–1% for the first point to 10–20% for the last point. Recently, the collection of data over a wider time range by superposition of different linear time scales was used for the resolution of complex decays (Szabo, A., personal communication). The collection of data using a logarithmic time base was the key

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to prove distributions of rates in proteins (2), and the use of this method in fluorescence spectroscopy should improve the resolvability of time domain measurements. In the frequency domain, data can be collected over three to four frequency decades. Generally, 20–40 data points are collected logarithmically spaced on the frequency axis. More important, the error of each phase and modulation point is on the order of 0.1% and is independent upon the frequency (3). The percent error refers to uncertainties of 0.1° in the phase measurement over a full scale of 90° and 0.001 in the modulation measurement over a maximum value of 1. These experimental uncertainties translate to errors of the same order of magnitude in the decay lifetimes when data are fitted to few exponentials. As a result of the improvement in accuracy and precision of frequency domain instrumentation, the resolvability of the decay has increased. In the past, data obtained with 1% error were often well fitted with a single exponential. Presently, data with 0.1% error of the same systems frequently require more than a single component to produce good fits. The problem of the resolvability of discrete components in the frequency domain has been studied by Lakowicz et al. (4). James et al. (5, 6) have pointed out that two exponentials can fit a variety of other decays. However, the problem of the resolvability of a single component has not been addressed. Continuous distributions of lifetime values can be fitted within the experimental error by a single exponential as shown below.

The discrete component analysis of the fluorescence assumes that all the radiating fluorophores decay with a well defined set of few lifetimes. In the case of heterogeneous systems in which the electronic environments of the emitting molecules are far from being unique and can change during the excited state lifetime such an approach becomes questionable. Two relevant examples of nonhomogeneous systems are the naturally fluorescent residues in proteins and probes in natural and synthetic membranes that exist in a variety of microenvironments. Here we first analyze continuous distributions of lifetime values using a sum of exponentials. In the case of a single exponential, we determined the maximum widths of symmetric distributions (uniform, gaussian, and lorentzian forms), which were indistinguishable from one exponential within the statistical uncertainty of 0.1% provided by current instrumentation. The same study was also conducted for an error of 1%. Similarly, the width limits of the above symmetric distributions, which can indistinguishably be fitted by two exponential decays, was established. In this case, we show that the discrete component analysis when used to study distributions of lifetimes was very sensitive to the number and range of frequencies at which the data are collected. In general a two exponential fit to a symmetric distribution yielded a nonsymmetric result: (*a*) the lifetimes obtained did not have symmetric values with respect to the center of the distribution, and (*b*) the relative fractions obtained did not have equal amplitudes. The result of the fit was symmetric only with very particular sets of frequencies whose values depended on the distribution shape. On the contrary, when a distribution was fitted by another distribution of different or equal functional form, the average lifetime value of the fit was largely insensitive to the set of modulation frequencies at which the data are obtained. The width from the fit was also representative of the real distribution width.

Finally, we analyzed asymmetric distributions and multimodal distributions using a superposition of symmetric functions. We show that the general shape of the lifetime distribution was recoverable regardless of (*a*) the functions used for the fit and (*b*) the set of frequencies at which the data are available. The resolvability was limited by the statistical uncertainty of the measurements.

Distribution of rates has been extensively considered in other fields, particularly in reaction kinetics and in low temperature flash photolysis. However, distribution of lifetime values has been introduced only recently in fluorescence (5, 6). Also different mathematical approaches have been used to recover the shape of the distribution of rates. Particularly important are the inversion procedures developed by Provencher and co-workers (7, 8).

METHODOLOGY

The fluorescence intensity is alternatively given by the following equations

$$I_F(t) = \sum_{i=1}^{n} a_i e^{-t/\tau_i} \tag{1}$$

$$I_F(t) = \sum_{i=1}^{n} f_i \tau_i^{-1} e^{-t/\tau_i}, \tag{2}$$

where n is the total number of components. In Eq. 1, a_i are the preexponential factors whose sum is usually normalized to one. In Eq. 2, f_i is the fractional contribution to the steady-state fluorescence by component ith. The relative values of both the preexponential factors and the light fractions as a function of lifetime can be determined by a distribution function. In this work we used Eq. 2, although both representations of the decay give similar results.

Simulated data of discrete and continuous distributions of exponentially decaying components were obtained as follows. Discrete distributions of components were generated using a set of exponentials evenly spaced in lifetime with amplitudes determined by the functional form of the distribution as illustrated in Fig. 1. The lifetime gaps between contiguous exponential components decrease proportionally to the number of components in the set. The distribution functions used in this work are listed in Table I. In general any distribution functional form can be generated in this manner. Continuous distributions of exponentials were generated by integration of the intensity Eqs. 1 or 2, which yield the fluorescence decay curve in the limit when a large number of components is used. However, from the computational point of view, a discrete

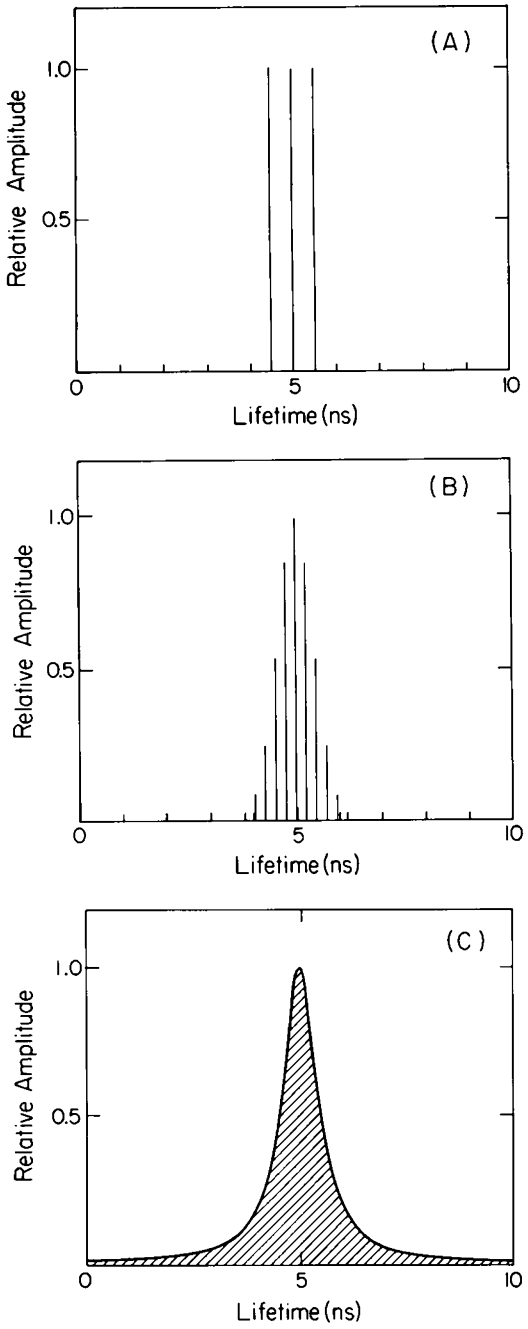

FIGURE 1 Distributions of equally spaced lifetime components centered at 5 ns with 1-ns widths. (*A*) Three exponentials in a uniform distribution, (*B*) 11 exponentials in a gaussian distribution, and (*C*) continuous lorentzian distribution.

distribution with several components per nanosecond lifetime interval gave the identical results as continuous distributions.

When a fluorescent system is excited by a sinusoidally modulated light intensity at an angular frequency ω given by

$$E(t) = E_0(1 + M_e \sin \omega t), \tag{3}$$

where E_0 is the average intensity and M_e is the modulation

TABLE I
DISTRIBUTION FUNCTIONS

Uniform	$f(\tau) = A$ from $C - W/2$ to $C + W/2$
	$f(\tau) = 0$ elsewhere
Gaussian	$f(\tau) = Ae^{(\tau - C)^2 \ln 2/4W^2}$
Lorentzian	$f(\tau) = A/\{1 + [(\tau - C)/(W/2)]^2\}$

C is the center, W the FWHM, and A a constant determined by the normalization condition

$$\int_0^\infty f(\tau)\, d\tau = 1.$$

The intensity decay is given by

$$I_F(t) = \int_0^\infty f(\tau)\tau^{-1}e^{-t/\tau}d\tau.$$

of the excitation, the response of the system can be written in the form

$$F(t) = F_0[1 + M_f \sin(\omega t - \phi)]. \tag{4}$$

F_0 and M_f are the average fluorescence and its modulation, respectively. The fluorescence is phase shifted with respect to the excitation by a value ϕ and demodulated such that the ratio $M = M_f/M_e < 1$. At a given modulation frequency, the measurable quantities ϕ and M are related to the physical parameters of the fluorescent population by the following equations (10):

$$\phi = \tan^{-1} S(\omega)/G(\omega) \tag{5}$$

$$M = [S^2(\omega) + S^2(\omega)]^{1/2}, \tag{6}$$

where

$$S(\omega) = \int_0^\infty I_F(t) \sin \omega t\, dt / \int_0^\infty I_F(t)\, dt \tag{7}$$

$$G(\omega) = \int_0^\infty I_F(t) \cos \omega t\, dt / \int_0^\infty I_F(t)\, dt. \tag{8}$$

$I_F(t)$ is given by Eqs. 1 or 2, and $S(\omega)$ and $G(\omega)$ are the sine and the cosine Fourier transforms of the fluorescence intensity $I_F(t)$. Phase and modulation data were generated using sets of components with amplitudes determined according to a given distribution function. Continuous distributions were then obtained using a sufficiently large number of components per unit lifetime interval. Different magnitudes of error were added to the phase and modulation values to simulate experimental data. The error added was frequency independent and defined as the standard deviation of several observations of the measured quantity. In phase fluorometry data are accumulated and averaged at a given frequency until the established standard deviation is obtained. The determination of the fluorescence decay in the frequency domain involves the measurement of the phase shift and the modulation ratio at a number of frequencies generally chosen to allow these quantities to gradually vary over the widest possible range. Usually a minimum of 8–12 frequencies (16–24 data points) are

sufficient to accurately determine the decay curve. The simulations presented in this paper were conducted using data at 25 frequencies in the 1–500-MHz range and with frequency values logarithmically spaced with a ratio of 1.28. The distribution models used to fit the data require the use of a nonlinear minimization algorithm. The function to be minimized is the reduced χ^2 defined as

$$\chi^2_R = \frac{1}{(2n - f - 1)} \sum \left[\frac{(\phi_m - \phi_c)^2}{\sigma^2_\phi} + \frac{(M_m - M_c)^2}{\sigma^2_M} \right], \quad (9)$$

where the calculated values of ϕ_c and M_c at frequency ω are given by Eqs. 5 and 6, ϕ_m and M_m are the simulated values of ϕ and M, n is the number of modulation frequencies, f the number of the parameters used for the fit, and σ_ϕ and σ_M are the standard deviation of phase and modulation measurements, respectively. For a good fit the value of the reduced χ^2 should be close to one. The characteristic decay parameters appear in Eqs. 5 and 6 through direct substitution of Eq. 2 into Eqs. 7 and 8. The simplex algorithm (10) was used to minimize the χ^2. An IBM personal computer with a 8087 coprocessor (IBM Instruments, Inc., Danbury, CT) was used for the calculations reported here. For all simulations the average value of the distribution was centered at 5 ns. When the ratio of the width to center is used to characterize the distribution, the analysis of the distributions becomes independent of (*a*) the particular average values around which they are centered, and (*b*) the particular widths.

RESULTS AND DISCUSSION

Single Exponential Fits to Distributions of Lifetime Values

Fig. 2 shows the variation of the reduced χ^2 of single exponential fits to continuous uniform distributions of components of variable width, centered at 5 ns. The statistical error was 0.1%. The shaded area represents the range of χ^2_R values obtained in the fits within 90% confidence interval. The zero width limit gives the range of χ^2 values for fits to a single exponential. On the basis of the reduced χ^2 values, uniform distributions of width-to-center ratio of <0.32 can yield indistinguishable results from single exponentials. However, a slight correlation of the residues seems to appear for width/center ratios larger than 0.2. Below this limit, uniform distributions of components and a single exponential decay become indistinguishable. When the number of frequencies at which the data are collected increases, the χ^2 curve similar to that in Fig. 2 becomes narrower, and the maximum width of uniform distributions indistinguishable from a single exponential is smaller. Studies performed at 16 frequencies yielded similar limits of resolvability. With four frequencies the width of the distribution indistinguishable from a single exponential substantially increased. Similarly, in the case of 1% statistical error, uniform distributions of exponentials of

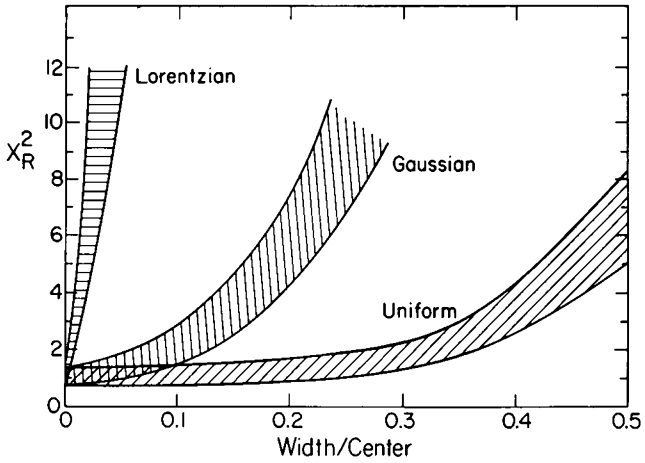

FIGURE 2 Reduced χ^2 surface of single exponential fits to uniform, gaussian, and lorentzian distributions of components centered at 5 ns with variable widths. The shaded area represents the 90% confidence values in the reduced χ^2 when data at 25 frequencies are used for the analysis and the error level was 0.1%. Data are plotted vs. width/center ratio.

widths up to 0.6 in center units are indistinguishable from single exponentials. The same study was conducted for uniform discrete distributions with statistical errors of 0.1%. The number of components per uniform distribution were 3, 21, and 41. Reduced χ^2 surfaces similar to those in Fig. 2 were constructed. The results obtained were practically indistinguishable from those obtained in the case of continuous uniform distributions. We concluded that in the case of a statistical error of 0.1%, single exponentials can fit uniform distributions of components with a width-to-center ratio of 0.2:0.3 regardless of the number of components in the set. Also the analysis of the residues show no correlation. The results obtained for the χ^2_R curve using the gaussian and the lorentzian functions to convolute the amplitude of the set of exponentials followed similar patterns as that of Fig. 2. The maximum width of the distribution that can indistinguishably be fitted with a single exponential was largely independent of the number of components used to generate the distribution. A single exponential decay can indistinguishably fit gaussian distributions of lifetimes with widths (in center units) <0.19 when the experimental error is 0.1% and 0.56 when the error is 1%. Similarly, a single exponential decay can indistinguishably fit a lorentzian distribution of lifetimes with maximum width/center ratio 0.01 when the statistical uncertainty of the data is 0.1% and with a maximum width/center ratio of 0.07 when the statistical error is 1%. As the distribution of lifetimes becomes more confined in smaller interval the width of the resolvable distribution increased and vice versa, as the distribution spreads in a larger lifetime interval the width of the distribution, indistinguishable from a single exponential decay, became narrower. The uniform distribution, which confines all the components in the smaller lifetime interval, required a larger width to be resolved. The lorentzian distribution,

which spreads in a large lifetime interval, yielded the lowest value. The gaussian, which spreads over an intermediate lifetime interval, also yielded a minimum resolvable distribution width between the uniform and the lorentzian. The relative spread of these distributions can be compared in Fig. 1 in which the three functions are centered at 5 ns and have the same full width at half the maximum of 1 ns.

Assuming experimental conditions in which systematic errors are absent and considering data that "perfectly" fit a single exponential decay, the question is to establish whether the data represent a sample decaying with a unique lifetime or a distribution of lifetimes. The uncertainty of the lifetime value obtained from a fit using single exponential does not directly relate to the width of the lifetime distribution. The uncertainty is determined by the curvature of the χ^2 surface and can be estimated using the covariance matrix of errors (4) and is of the same order of the statistical error. For a lifetime distribution centered at 5 ns and of 2.5 ns width, the lifetime uncertainties obtained for a fit to a single exponential were 4 and 30 ps for statistical errors of 0.1 and 1%, respectively. Those values obtained from the covariance matrix of errors are independent upon the distribution width, and they must be considered as the uncertainties of the determination of the average value rather than being representative of the width of the distribution. Even though both models may fit experimental data indistinguishably, their physical implications are quite different. The single exponential approach assumes that all fluorophores of the sample population decay with a unique rate. Such a hypothesis can be regarded as very improbable for fluorophores in solution in which the interactions with other molecules (collisions, relaxations, etc.) affect the excited state lifetime and, specifically, for fluorophores in nonhomogeneous environments. Rather, the lifetime of a fluorophore in an environment that varies from site to site can be defined by probability density functions that yield the probability of finding the fluorescence lifetime of a molecule of the population within a lifetime interval. The uncertainty in its determination is then given by a characteristic parameter of the function (i.e., FWHM, standard deviation, etc.) used to fit the data. Alternatively, the fluorescence of a sample can be regarded as due to a large set of single exponential decays each related to a particular environment of the fluorophore. Lifetime distribution functions yield the fraction of components per lifetime interval.

A distinction exists between probability density functions and lifetime distribution functions. Any function that fits the data reasonably well can in principle be used as a probability density function. A variety of these functions yields comparable results when the probability of finding the lifetime within a given interval is the goal. Lifetime distribution functions are expected to yield the real distribution of lifetimes of the molecules of the sample. In this case the functions should be derived from physical grounds. When a single exponential is used to fit either a non-well defined decay or a distribution, the result obtained gives only the average lifetime value.

Double Exponential Fits to Distributions of Lifetime Values

Two-exponentials fits to simulated data at 25 frequencies with 0.1% error of the uniform, gaussian, and lorentzian distributions as a function of the width-to-center ratio were conducted. The maximum widths in center units of these distributions that were indistinguishable from two exponentials based on the reduced χ^2 and on the analysis of the residues were 1.0, 0.5, and 0.015 for the uniform, gaussian, and lorentzian distributions, respectively. Fig. 3 shows the two component analysis of the respective continuous distributions. The widths of the distributions shown in the respective figures correspond to the maximum values they can have being indistinguishable from the two exponential components analysis. The shorter lifetime component always had the smaller contribution. Such behavior is due to a systematic effect. In general a two-exponentials fit to simulated data from a symmetric distribution is expected to give two components of equal amplitudes with lifetimes symmetrically located with respect to the original distribution. The asymmetric results of Fig. 3 were primarily due to the set of frequencies used that weighted the components of the distribution not uniformly. For a given distribution function the sets of frequencies that weight all the components of the distribution uniformly must be chosen depending upon the shape of the particular distribution. There is no standard set of frequencies that uniformly weights all the components of the distribution regardless of its shape. To illustrate this statement consider the phase shift and modulation ratio as a function of frequency for a single exponential, which is given by Eqs. 10 and 11

$$\phi = \tan^{-1} \omega\tau \tag{10}$$

$$M = [1 + \omega^2\tau^2]^{-1/2}. \tag{11}$$

The lifetime of a single component is most accurately determined when the phase shift is 45 degrees (modulation ratio of $1/\sqrt{2}$). The frequency at which this situation occurs is $\omega = 1/\tau$. In the case of distributions, the optimum frequency can then be determined for each lifetime component. For a given lifetime set, an optimum modulation frequency distribution can be generated as shown in Fig. 4 in which each component with its amplitude is plotted at the frequency at which it is best determined. Instead, in a typical experiment the set of frequencies used are evenly spaced in a logarithmic scale, which is symmetric for the phase and modulation curves. Fig. 4 shows the optimum frequency distribution, and it is apparent that sets of components evenly spaced in lifetime do not yield an evenly spaced set of optimum frequencies in the logarithmic

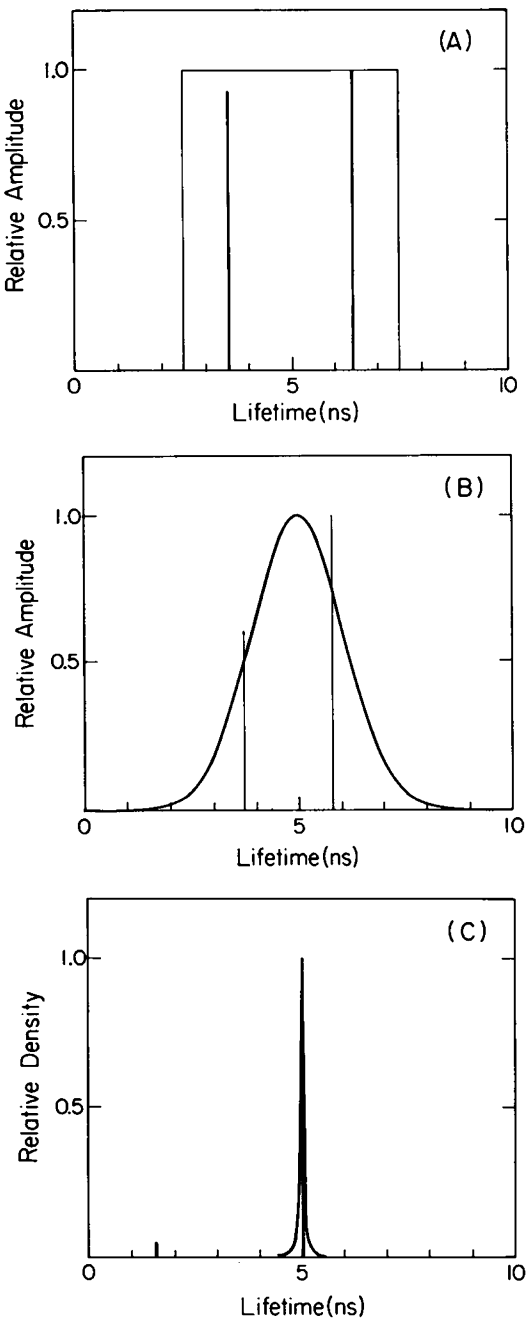

FIGURE 3 Two component analysis of distributions at the limit of resolvability. (*A*) Uniform, (*B*) gaussian, and (*C*) lorentzian.

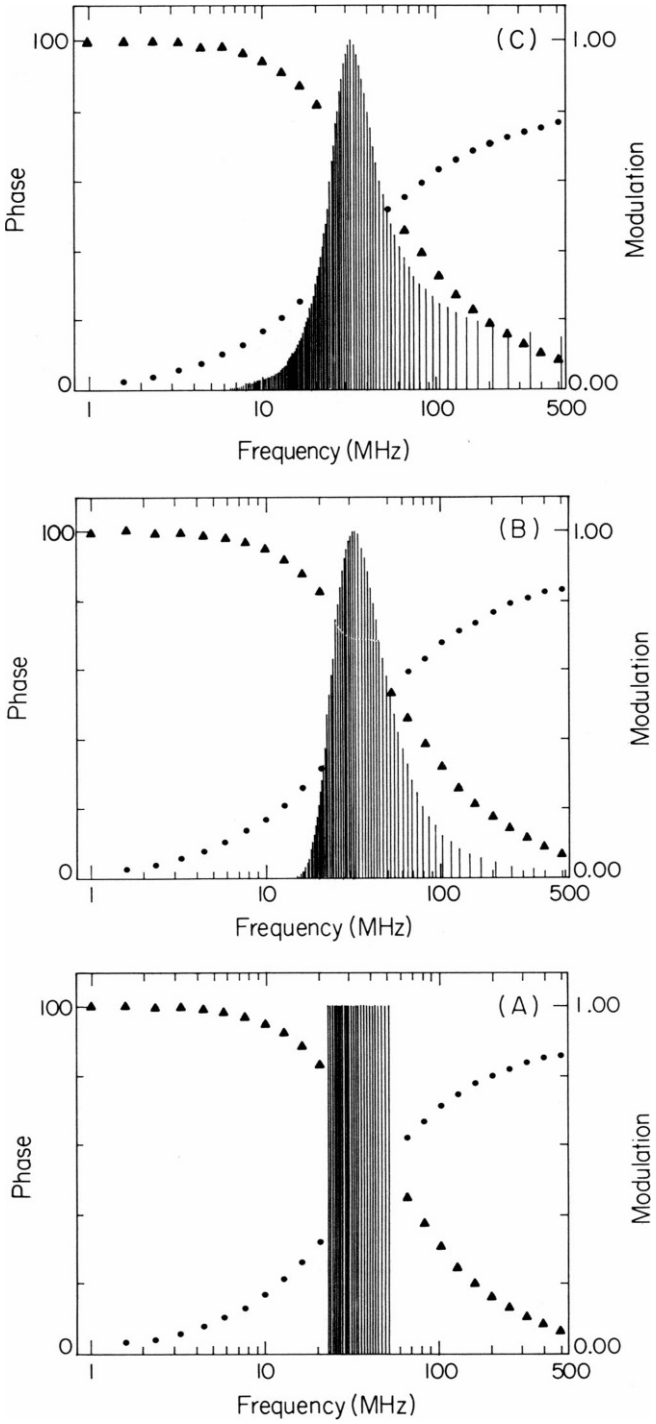

FIGURE 4 Optimum frequency sets of lifetime distribution functions. (*A*) Uniform, (*B*) gaussian, and (*C*) lorentzian. The phase and modulation data correspond to the respective distributions. Sets of components evenly spaced in lifetime do not yield evenly spaced sets of optimum frequencies in the logarithmic frequency axis.

frequency axis. When a distribution was used, the fit did not uniformly weight all the components. For example consider the worst case given by the lorentzian lifetime distribution. The shorter components that extend to zero lifetime were not represented by data at high enough frequencies, which caused the two exponential analysis to fit the longer lifetime component with the largest amplitude. The same effect occurred, with less emphasis, in the gaussian and uniform distributions. However, while in the case of the latter distributions it is usually possible to collect data in the 1–500-MHz frequency range to obtain a symmetric two component fit, in the case of the lorentzian distribution it is impossible. Data at higher frequencies than those available in current instrumentation are needed.

TABLE II
ANALYSIS DATA FROM A GAUSSIAN DISTRIBUTION CENTERED AT 5 NS AND OF 2.5-NS FWHM

	Frequency range	Lifetime	Fraction	χ^2
	MHz	*ns*		
Two-exponentials analysis	1–10	6.82 ± 11.53	0.208 ± 0.203	0.90
		4.81 ± 2.8	0.792	
	100–500	5.78 ± 0.73	0.723 ± 0.329	0.92
		3.67 ± 0.84	0.272	

	Frequency range	Center	Width	χ^2
	MHz	*ns*	*ns*	
Uniform distribution analysis	1–10	5.09	2.69	0.85
	100–500	5.00	2.86	0.86

Distribution Fits to Distributions of Lifetimes

The situation is different when probability density functions (and lifetime distributions) were used. In this case the nonsymmetric factors practically cancel out. The probability functions or the lifetime distribution are weighted in a similar way by the set of frequencies used. In Table II the data fitted were in the 1–10-MHz and 100–500-MHz range. In both cases the frequency range was located outside the optimum frequency range described above. Table II shows also the lifetime values recovered using a two-components analysis, which yielded large uncertainties in the lifetime values and asymmetric results. The fit using a uniform distribution yielded good fits, which were also representative of the original distribution. An advantage of using arbitrary probability functions to fit the decay from a set of components is that the real lifetime distribution can be recovered when adequate functions are used. Fig. 5 shows data generated from two gaussian distributions of components fitted using two uniform functions. In Fig. 5 *A* the two gaussian distributions are well separated and the two uniform probability functions used to fit the data recovered a bimodal distribution with centers and widths representative of the original distribution. As the centers of the two gaussians come closer, the fit using the bimodal uniform probability function adjusted its parameters to best account for the shape of the real distribution. Notice in Fig. 5, *B–D*, the superposition of the two uniform functions that follow the shape of the lifetime

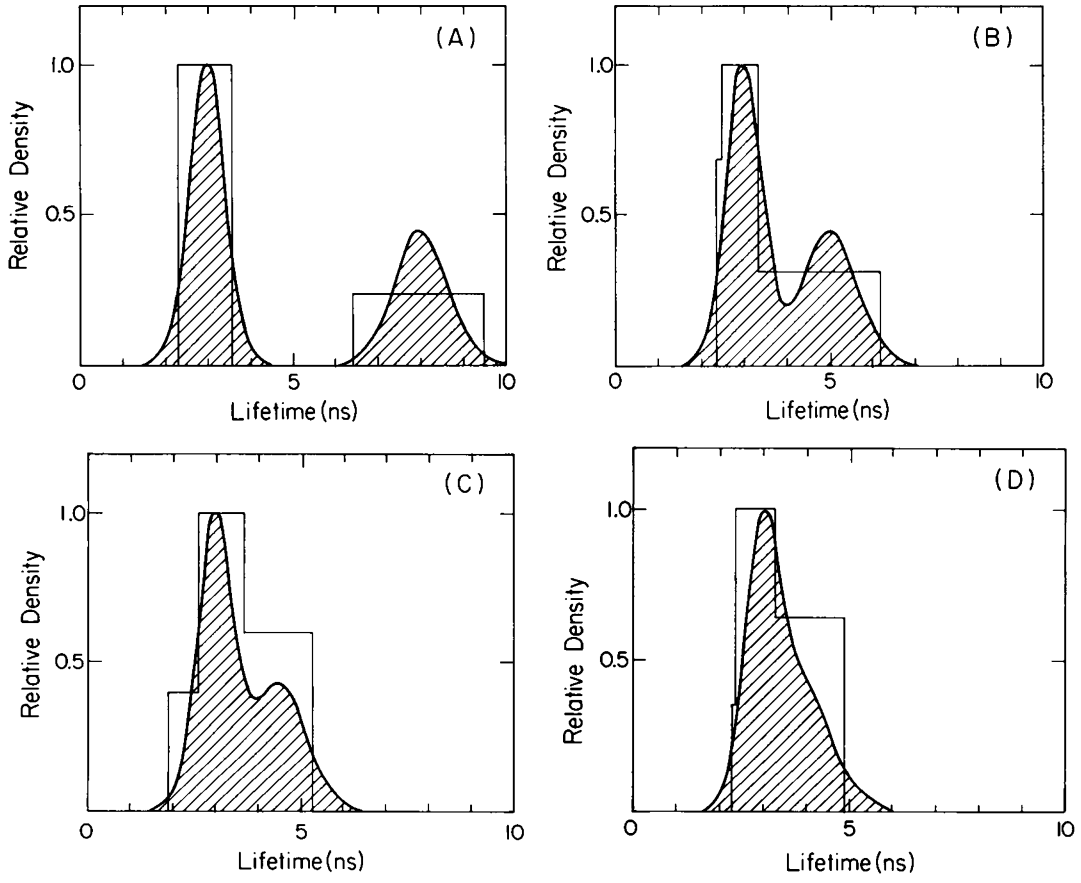

FIGURE 5 Recovery of lifetime distributions using probability density functions. Data generated from the shaded distributions of components (two gaussians) were fitted to a bimodal probability function (two uniform distributions). *A–D* show a few cases. In all cases the values of the χ^2 were ~1.

component set. The recovery of the original distribution depended only upon the statistical error of the data and was largely independent on the position and shape of the distribution.

GENERAL RESULTS

In what follows we present some general results from our experience in fitting arbitrary functions to experimental and simulated data. (*a*) When data from a single symmetric distribution of lifetime values was fitted using two or more symmetric functions, the fraction of only one component was different from zero and the additional components vanished. As an example here we mention the fit to experimental data from the fluorescence decay of the protein renilla. Three gaussian distributions with variable fractions and variable widths and centers were used. The result of the fit yielded two fractions with zero contribution. When two gaussian distributions were used for the fit one fraction was zero. Finally, when one gaussian distribution was used the best fit was obtained. Similar results were observed using uniform and lorentzian functions. (For this protein the discrete component analysis yielded 98% of one component.) (*b*) When bimodal distributions were fitted with one symmetric function, the result was a poor fit and a large width for the function (Fig. 6). The outcome of using the superposition of three distributions generally reduce to either a negligible fractional contribution of one of them or to two distributions with very close center values. Two symmetric functions recovered a distribution form similar to the original bimodal distribution as shown in Fig. 5. (*c*) The general shape of an arbitrary lifetime distribution can be proved using multimodal probability density functions with one restriction. The ratio width/center of each function member of the multimodal probability function should have a larger value than the minimum value determined by the ratio, which cannot be distinguished from a single exponential. When this criteria was not followed, the fits gave a variety of solutions with distribution widths less than the minimum resolvable. (*d*) For a wide variety of probability density functions there is a maximum moment beyond which larger moments of the function are not determined. In general, the shape of a lifetime distribution can be recovered using multimodal probability density functions if both functions have comparable maximum moments. For example, the gaussian and the uniform functions define an infinite set of moments and (within the resolvability limits previously reported) multimodal gaussian distributions can be recovered using multimodal uniform functions (and vice versa). In the case of the lorentzian function all moments, except the zero moment, diverge. Lorentzian functions cannot be used to recover the shape of lifetime distributions, which have defined moments. An extreme case is illustrated in Fig. 7 in which the bimodal gaussian distributions of Fig. 5 are fitted to bimodal lorentzian distributions. In this case, the widths and centers of the fit do not represent those of the original

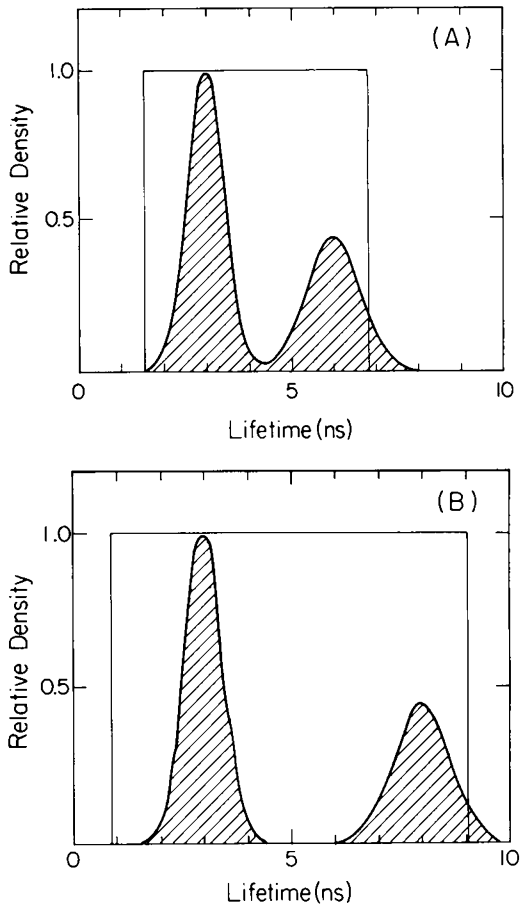

FIGURE 6 Bimodal distribution fitted by a single probability function. The values of the reduced χ^2 were considerably larger than 1.

distribution. Additionally, the fits obtained gave poor χ^2_R values.

A note of warning should be given here when two and three distributions are used for the fit. Two symmetric functions involve five independent fitting parameters. In this case partial minima can sometimes be found by the minimization algorithm. Three symmetric functions involve eight independent fitting parameters and partial minima are frequently encountered. In both cases it is suggested that the optimum value be obtained by exploring the entire χ^2 surface. Most of the partial minima can be avoided when the ratio width/center of the fitting functions are restricted within the limits of physical significance determined by statistical error.

CONCLUSIONS

A "good" fit of the fluorescence decay curve to single exponential does not necessarily imply that the fluorescence is generated by one component. Narrow lifetime distributions can be fitted to a single exponential within the statistical error provided by present instrumentation. The single exponential observed in high pressure gasses, liquids, and amorphous solids in which the environment of radiating fluorophores is not unique can rarely be expected to be

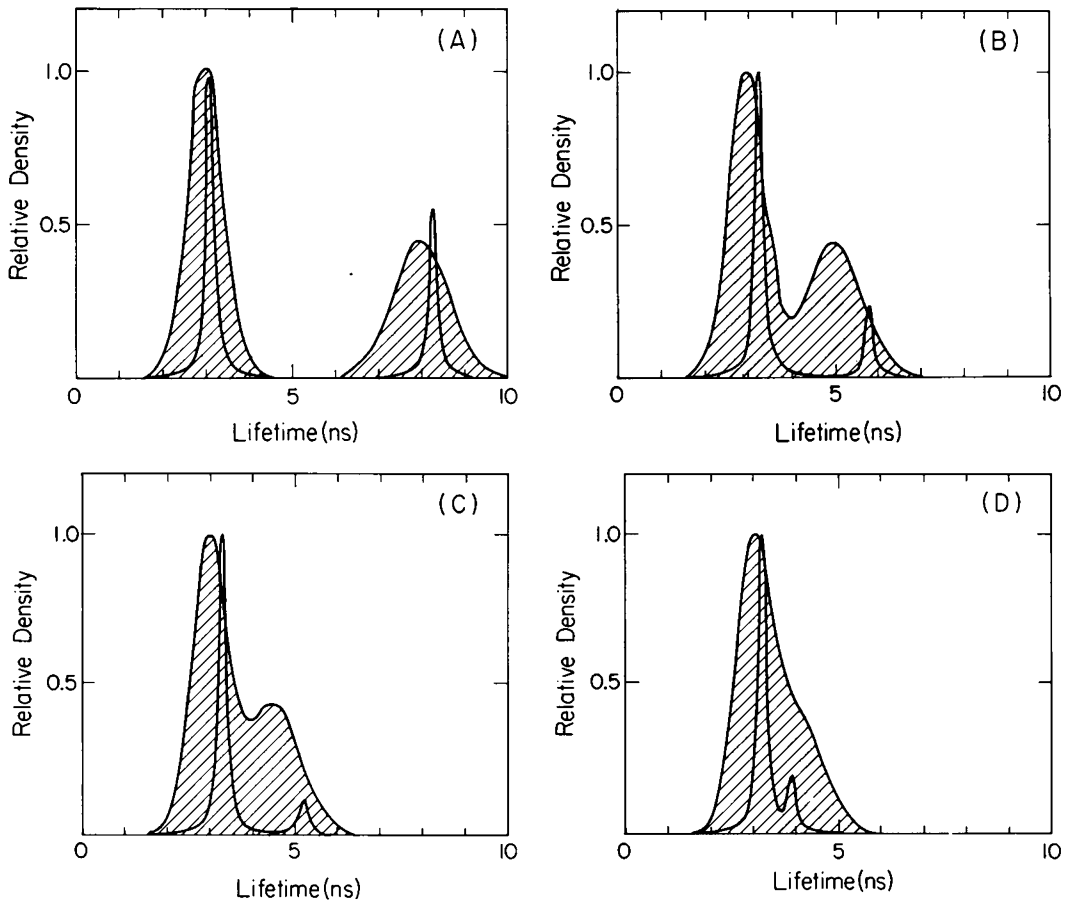

FIGURE 7 Fit of bimodal gaussian distributions (the gaussian distribution has an infinite set of moments) using bimodal lorentzian probability density function (the moments of the lorentzian distribution diverge). The reduced χ^2 values obtained are large and the distributions are not recovered.

due to one decay rate. Rather it is more reasonable to assume a narrow distribution of decay rates, which can indistinguishably be fitted by one exponential. Narrow distributions also arise in heterogeneous systems when the rate of interconversion between different environments is faster than the decay of the excited state. Broader distributions of components can also indistinguishably be fitted using more than one exponential. However, this approach does not weight uniformly the real distribution, and the result of the analysis is dependent upon the set of frequencies at which the data were collected. The analysis of the data in terms of probability density and lifetime distribution functions was less sensitive to systematic errors due to the weighting of the data. A distribution of lifetimes can be probed using multimodal probability density functions. The resolvability of this approach is limited by the statistical uncertainty of the measurements. Further improvements in the accuracy and precision of the instrumentation will favor the study of distributions and their relation to the molecular details and dynamics of the surroundings. The physical significance of the width of the lifetime distribution must be related to processes and mechanisms that can modify the lifetime of a molecule, in particular the effect of the microenvironment of each molecule. This new approach can have wide applications for the analysis of the fluorescence from a large number of biological systems where the conditions to generate a distribution of rates are likely to exist. In these cases, the average lifetime as determined by the conventional analysis of the decay gives only the lowest moment of the distribution. In the companion paper that follows (11) lifetime distribution functions that are compatible with the observed fluorescence decay of tryptophan residues in proteins are derived from physical grounds. The third companion paper (12) shows the fits of these distribution functions to actual data and relates them to the protein structure and dynamics.

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