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Phase fluorometric method for determination of standard lifetimes.

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mixtures of analytes. This aspect is to be investigated.

Although the use of the photochemical reactor is limited to phenolic dansyl derivatives, the technique can be very useful in the reversed-phase LC analysis of drugs with phenolic groups and many possible metabolites as well as other biologically interesting compounds, e.g., catecholamines, estrogens, and thyroid hormones. In addition, it may have a high potential for studies on the metabolism of xenobiotics such as polycyclic aromatic hydrocarbons (21), biphenyls (11), halogenated aromatic hydrocarbons (22, 23), etc., where aromatic hydroxylation often is a major metabolic pathway.

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Registry No. Phenol, 108-95-2; 2-chlorophenol, 95-57-8; 3-chlorophenol, 108-43-0; 4-chlorophenol, 106-48-9; 4-fluorophenol, 371-41-5; 4-bromophenol, 106-41-2; 4-nitrophenol, 100-02-7; 4-methoxyphenol, 150-76-5; 4-ethylphenol, 123-07-9; 2,3-dichlorophenol, 576-24-9; 2,4-dichlorophenol, 120-83-2; 2,5-dichlorophenol, 583-78-8; 2,6-dichlorophenol, 87-65-0; 3,4-dichlorophenol, 95-77-2; 3,5-dichlorophenol, 591-35-5; 2,3,4-trichlorophenol, 15950-66-0; 2,3,5-trichlorophenol, 933-78-8; 2,3,6-trichlorophenol, 933-75-5; 3,4,5-trichlorophenol, 609-19-8; 2,4,5-trichlorophenol, 95-95-4; 2,4,6-trichlorophenol, 88-06-2; 2,4,6-tribromophenol, 118-79-6; 2,3,4,5-tetrachlorophenol, 4901-51-3; 2,3,4,6-tetrachlorophenol, 58-90-2; 2,3,5,6-tetrachlorophenol, 935-95-5; pentachlorophenol, 87-86-5; 2,3-dimethylphenol, 526-75-0; 2,5-dimethylphenol, 95-87-4; 2,6-dimethylphenol, 576-26-1; 3,4-dimethylphenol, 95-65-8; 3,5-dimethylphenol, 108-68-9; 1,2-dihydroxybenzene, 120-80-9; 1,3-dihydroxybenzene, 108-46-3; 1,4-dihydroxybenzene, 123-31-9; water, 7732-18-5.

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Phase Fluorometric Method for Determination of Standard Lifetimes

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Rayleigh scatterers have long been used as standards for fluorescence lifetime determinations, but they have many drawbacks, including the well-known "color effect". To avoid these problems, various fluorophores have been used as standards. Unfortunately, the lifetimes of these compounds are not agreed upon to better than 5%, and the compounds cited in the literature do not fully cover the 250-850 nm band of common fluorescence emission. We describe a multifrequency phase fluorometric method for accurately determining the lifetimes of monoexponential fluorophores (standards) without reference to another standard. Results are shown for some widely used standard fluorophores and some recently developed compounds. An independent test of the accuracy of the method based on quenching experiments is presented.

Fluorescence emission decay kinetics have long been a useful

tool for studying a variety of chemical, physical, and biological systems. Like any other measurement of this sort, a standard is necessary for calibration of fluorescence lifetime instrumentation, and for determining the instrument response function. The most important early standards were Rayleigh scatterers, which are convenient and have a "lifetime" that is zero, and thus accurately known. Unfortunately, scatterers have a number of well-known drawbacks, including the "color effect" and other optical artifacts, which limit their usefulness. The color effect arises because the transit time for photoelectrons through the photomultiplier varies with the wavelength of the light hitting the photocathode, and consequently, the scattered light and the fluorescence can have different delays. Recognizing this, Wahl (1) and others (2-7) introduced various fluorophores as standards, hoping to match the sample (often protein) emission wavelength as closely as possible and minimize these artifacts.

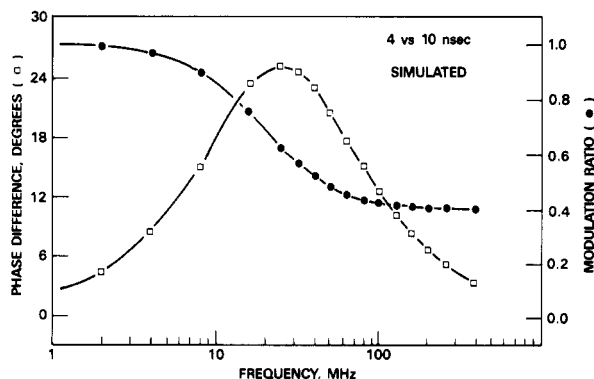


Figure 1. Simulated frequency-dependent phase differences (\square) and demodulation ratios (\bullet) between 10- and 4-ns monoexponential decays. At 40 MHz, the phase angle of a 10-ns decay is 68.3° and of a 4-ns decay is 45.2° ; the difference is 23.1. Similarly, at 40 MHz the demodulations of 10- and 4-ns decays are 0.370 and 0.705, respectively, and their ratio is 0.524.

Unfortunately, problems remain with using the published values for current lifetime standard fluorophores. Most important, there is no agreement in the literature (to better than 5%) on the lifetime of any common standard. Clearly, this sets a limit on the accuracy of any determination, despite the fact that the precision of current multifrequency phase and time-correlated single photon-counting instrumentation is perhaps 0.5% or better (8, 9). Moreover, these discrepancies may not reflect inaccurate measurements, but rather the difficulty of preparing identical reference solutions—particularly controlling the concentration of the ubiquitous quencher, oxygen. Another difficulty lies in the limited number of standard compounds that have been promulgated in comparison to the wide spectral range of current interest: from 250 to 850 nm.

While a transferable standard material would be convenient, it is not absolutely necessary in this case. What is required is a *method* to measure the (monoexponential) decay of a standard fluorophore under laboratory conditions with great accuracy. The method we describe employs multifrequency phase fluorometry to do this, without reference to any standard material or lifetime value. The basis of the method is described, together with its application to widely used standards and recently introduced compounds.

THEORY

For a fluorophore which decays exponentially with lifetime τ , the values of the phase and modulation are given by

$$\phi = \tan^{-1}(\omega\tau) \quad (1)$$

$$m = \frac{1}{\sqrt{1 + \omega^2\tau^2}} \quad (2)$$

where ω is the angular frequency of light modulation. The values of the phase difference and modulation ratio between two fluorophores which decay exponentially are given by

$$\Delta\phi = \tan^{-1}(\omega\tau_1) - \tan^{-1}(\omega\tau_2) \quad (3)$$

$$\frac{m_1}{m_2} = \sqrt{\frac{1 + \omega^2\tau_2^2}{1 + \omega^2\tau_1^2}} \quad (4)$$

A plot of the phase difference and modulation ratio as a function of the frequency for such a system is reported in Figure 1 for $\tau_1 = 10$ ns and $\tau_2 = 4$ ns. The phase plot is the difference between two arctangent functions, each with a 45°

point at a frequency $\omega = 1/\tau$. If the lifetime values are well separated, the phase difference will be large and the two lifetimes can be accurately determined. The frequency where the phase difference reaches a maximum is

$$\omega_m = \left(\frac{\tau_1 + \tau_2}{2} \right)^{-1} \quad (5)$$

and the maximum value of the phase difference is

$$\Delta\phi_m = \tan^{-1} \sqrt{\frac{\tau_1}{\tau_2}} - \tan^{-1} \sqrt{\frac{\tau_2}{\tau_1}} \quad (6)$$

For a ratio $\tau_1/\tau_2 = 2$, the maximum phase difference is approximately 20° . The peak value of the phase difference and its position in frequency space are thus respectively functions of the ratio (eq 6) and sum (eq 5) of the two lifetimes. Therefore a phase difference plot like Figure 1 is *uniquely* determined by the two lifetimes, and they may be recovered from such a data set.

The modulation ratio plot has a sigmoidal shape, starting at 1 at low frequency and decreasing to an asymptotic value of τ_2/τ_1 at very high frequency.

The uncertainty in the determination of τ_1 and τ_2 depends on the ratio τ_1/τ_2 , provided that the phase difference and modulation ratio can be determined with the same accuracy at all modulation frequencies. We have estimated that the absolute uncertainty when the ratio τ_1/τ_2 is very different from one is twice the uncertainty that can be obtained if one of the lifetimes is known exactly, which is of the order of 1–2 ps with present day instrumentation (8). For a ratio of 2 the uncertainty is about 5-fold larger than for the ideal case. When τ_1/τ_2 is only 1.1, the uncertainty is a factor of 100 greater, and if the ratio equals one, the lifetimes cannot be determined at all.

EXPERIMENTAL SECTION

Materials. Fluorophores and ethanol were of the highest purity commercially available and were not further purified. Water was double distilled. Dimethyl-1,4-bis(5-phenyl-2-oxazolyl)benzene (Me_2POPOP) was from Baker; 9-cyanoanthracene, rubrene, and Rose Bengal were from Aldrich; 70-pass zone refined anthracene was from CTC Organics, Atlanta, GA; Rhodamine 6G was from Exciton; glycogen was from Merck; and 6-methoxy-*N*-(3-sulfopropyl)quinolinium (MSQ) (10), Sulforhodamine 101, and *N*-(3-sulfopropyl)acridinium (SPA) were from Molecular Probes, Eugene, OR. Solvents showed negligible background fluorescence under the spectral conditions of the experiments, and no attempt was made to remove dissolved oxygen.

Instrumentation. Multifrequency phase fluorometric measurements were performed on an ISS Greg-200 (ISS, Inc., Champaign, IL) essentially as previously described (8, 11). Excitation was provided by a Liconix 4214NB HeCd laser at 442 and 325 nm. Appropriate filters were used to eliminate Rayleigh and Raman scatter from the emission. Excitation and emission spectra were obtained on a Spex Fluorolog II and are corrected for the wavelength dependence of the 450-W xenon arc excitation, but not for the wavelength dependence of the detection system; excitation spectra closely matched absorption spectra taken on a Perkin/Elmer Lambda 4C-HP spectrophotometer.

Method. The measurements were performed on pairs of putatively monoexponential materials (standards) using the double comparison format introduced by Spencer and Weber (12). In the example shown in Figure 1, the longer lifetime (10 ns) is the "sample", and the shorter (4 ns) is the "reference". The data collected are the phase differences and demodulations between the two monoexponential decays. Note that this differs from the usual procedure, in that we have not assumed a "standard" lifetime value for either one of the pair. These data are then fit to a pair of monoexponentials by using eq 3 and 4, with the usual criteria of low X^2 and random residuals being used to judge goodness of fit.

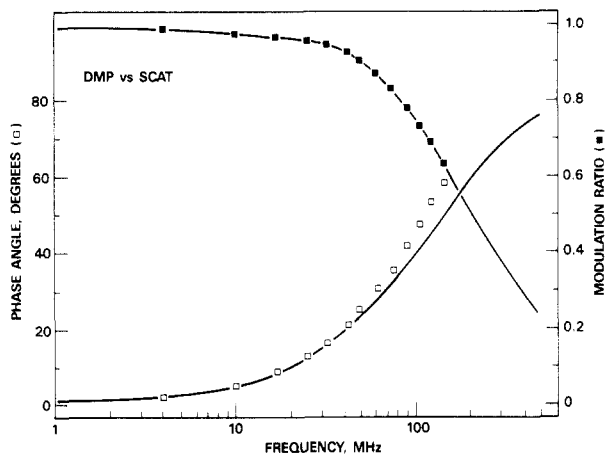


Figure 2. Frequency-dependent phase (\square) and modulation (\blacksquare) data for Me_2POPOP in ethanol versus a glycogen scatterer in water. The lines indicate the best fit to the modulation data alone. Excitation was at 325 nm, and the emission was viewed through a 0-52 Corning filter, but with no polarizer.

RESULTS AND DISCUSSION

Artifacts in Phase Fluorometric Measurements. It is worthwhile to depict a common artifact that can be introduced into multifrequency phase fluorometric measurements performed using a scatterer; one such data set is shown in Figure 2. We chose an extreme example of such artifacts for illustrative purposes. The existence of an artifact is shown by the good fit to a monoexponential by the modulation data alone (1.56 ns, $X^2 = 1.04$) but a much poorer fit to the phase data alone (1.925 ns, $X^2 = 60.3$) or both taken together (1.649 ns, $X^2 = 20$). It is apparent from the figure that there is a systematic phase error that increases with frequency. We note that although these data are consistent with a timing error, they are not necessarily the result of a color effect, since the effect does not increase monotonically with Stokes' shift, and the photomultipliers used here (Hamamatsu R928) are known to have a very small color effect (13). These effects seem to be more apparent with (highly polarized) laser excitation and can be decreased to some extent by the use of an emission polarizer at the "magic angle". This is because the emission from the standard is completely depolarized (rotational rate \gg emissive rate) whereas the scattered exciting light is completely polarized. Experience in our and other laboratories (B. Valeur and D. Jameson, personal communications) suggests that artifacts may be minimized when the emissions from sample and reference, and thus the optical paths leading to the detector for both, are as similar as possible.

Form of the Two Monoexponential Data Set. The form of the frequency-dependent phase differences and demodulation ratios expected for two monoexponential decays is shown in Figure 1. At 40 MHz, the phase angle of a 10-ns decay is 68.3° and of a 4-ns decay is 45.2° ; the difference is 23.1° . Similarly, at the same frequency the demodulations of 10- and 4-ns decays are 0.370 and 0.705, respectively, and their ratio is 0.524. In general the data will always have this form, as can be seen from the Theory.

The simulated data shown in Figure 1 in fact are uniquely defined by the two monoexponentials. This can be seen in Figure 3, which depicts simulated data for two pairs of monoexponentials having the same ratio of lifetimes, 0.95:1. The phase difference data from each pair in Figure 3A describes a curve having the same maximum phase difference of 1.4° , but shifted in frequency space. Similarly, the modulation ratios in Figure 3B describe identical curves offset in frequency space. Note that the curves in Figure 3B asymptotically approach the value 0.95, which is the ratio of lifetimes—as shown in Theory.

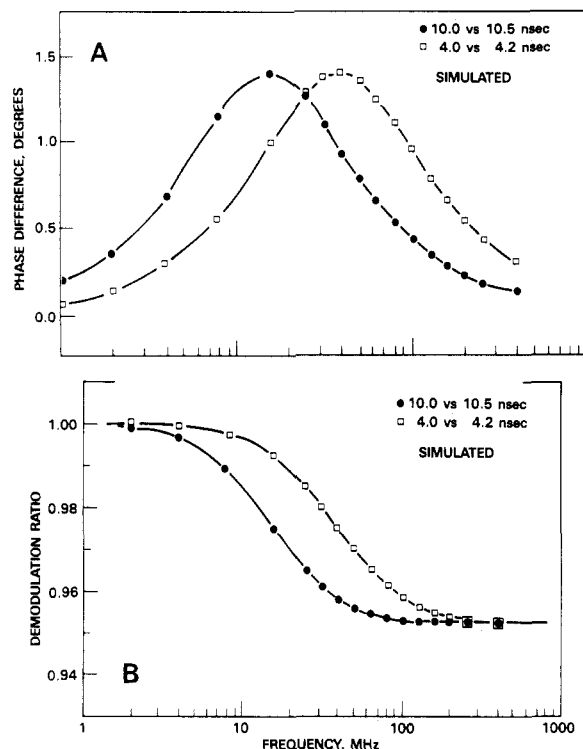


Figure 3. (A) Simulated frequency-dependent phase differences for two pairs of monoexponential decays. Data are shown for 4.0 vs 4.2 ns (\square) and 10 vs 10.5 ns (\bullet). (B) Simulated frequency-dependent demodulation ratios for two pairs of monoexponential decays. Data are shown for 4.0 vs 4.2 ns (\square) and 10 vs 10.5 ns (\bullet).

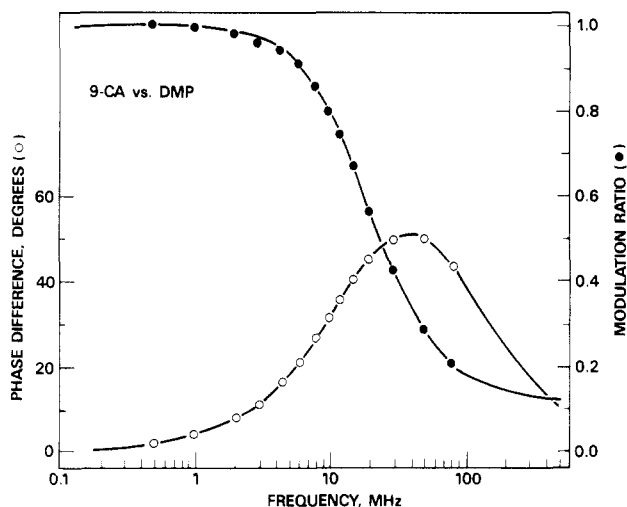


Figure 4. Measured frequency-dependent phase differences (\circ) and demodulation ratios (\bullet) for 9-cyanoanthracene vs Me_2POPOP , both in ethanol at 25°C . The lines indicate the best fit to the data, which were lifetimes of 11.94 and 1.49 ns, and $X^2 = 1.80$. Excitation was at 325 nm, and emission was viewed through a Corning 0-52 filter.

Experimental Results. We acquired data from pairs of fluorophores whose decays are believed to be monoexponential, and which had similar spectral characteristics. Such a data set is depicted in Figure 4 for 9-cyanoanthracene vs Me_2POPOP , together with the best two monoexponential fit. The fit to two monoexponentials is clearly good, as judged by the low X^2 (1.80), random residuals (data not shown), and the recovery of values for the lifetimes that are in good agreement with values reported in the literature (3, 6) and measured in this laboratory with a scattering solution (Table I). Results using this method are shown for other fluorophores in Table I, together with the best one-component fits obtained for the same compounds by using a scattering solution in the ordinary

Table I. Standard Lifetime Values

compound/solvent ^a	vs scatterer		2 mono-exponent	
	τ , ns	X^2	τ , ns	X^2
Rhodamine 6G/E	3.890 ± 0.014	2.26	3.740	0.75
Rose Bengal/E	0.752 ± 0.003	3.43	0.776	0.75
Me ₂ POPOP/E	1.672 ± 0.004	4.10	1.494	1.80
9-cyanoanthracene/E	11.73 ± 0.039	2.23	11.94	1.80
anthracene/E	4.337 ± 0.018	3.76	4.434	3.11
MSQ/W	26.25 ± 0.073	4.24	26.57	3.11
Rhodamine 6G/E	3.890 ± 0.014	2.26	3.959	2.15
Sulforhodamine/W	4.173 ± 0.018	3.13	4.351	2.15
SPA/W	31.56 ± 0.146	9.00	32.78	3.27
Rubrene/E	10.26 ± 0.072	2.66	10.83	3.27

^a Abbreviations: MSQ, methoxysulfo-propylquinolinium; SPA, *N*-(3-sulfo-propyl)acridinium; E, ethanol; W, water. For each compound, data sets were obtained versus a glycogen scatterer and fit to a single exponential, and versus another compound in this list with similar spectral characteristics and fit to a pair of monoexponentials.

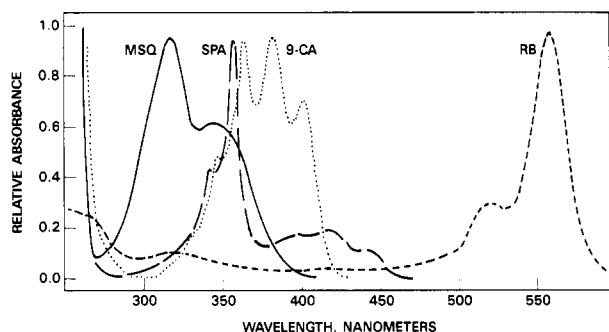


Figure 5. Normalized absorption spectra of 6-methoxy-*N*-(3-sulfo-propyl)quinolinium (MSQ) in water (—), *N*-(3-sulfo-propyl)acridinium (SPA) in water (---), 9-cyanoanthracene (9-CA) in ethanol (···), and Rose Bengal (RB) in ethanol (-·-·).

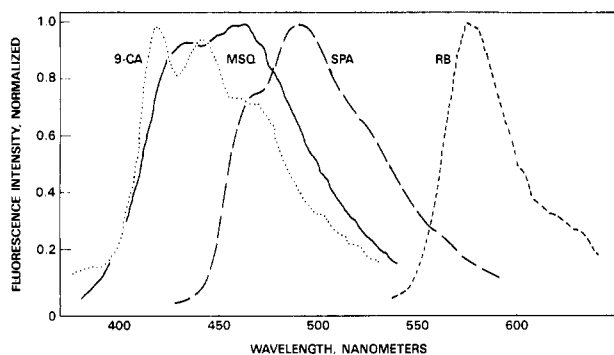


Figure 6. Normalized emission spectra of MSQ in water (—), SPA in water (---), 9-CA in ethanol (···), and RB in ethanol (-·-·). Excitation for MSQ and 9-CA was at 325 nm and for RB and SPA was at 442 nm.

way; absorption and emission spectra for selected fluorophores are depicted in Figures 5 and 6, respectively. In all cases, the best two-component fits to data sets obtained with a scattering solution yielded a major component comprising at least 97% of the total with a lifetime close to that obtained with the one component fit and a minor component with a very uncertain and/or nonsense lifetime; these are characteristics of a monoexponential decay fit with a two-component model (11).

The data generally show good agreement between the lifetime values obtained by using the two monoexponential method and using a scatterer or found in the literature (2–6, 14, 15). As is seen for data obtained by using a scatterer or reference compound (8, 11), poorer fits are obtained with the two-monoexponential algorithm when (a) an inadequate

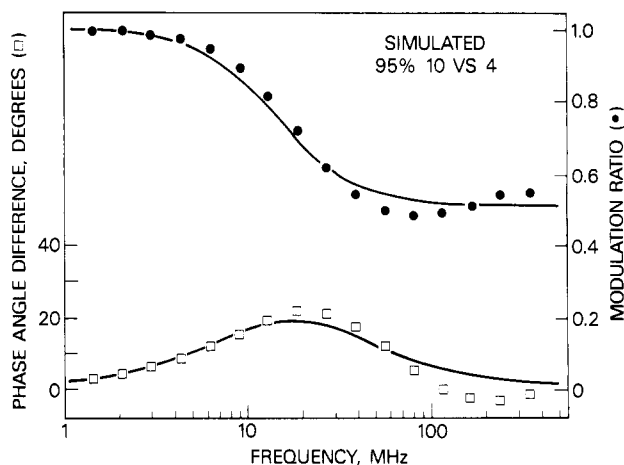


Figure 7. Simulated frequency-dependent phase differences (□) and demodulation ratios (●) for a mixture of 95% 10-ns emitter and 5% 1-ns emitter vs 100% 4 ns. The lines indicate the best two-monoexponential fit to the data, recovering lifetimes of 12.6 and 6.4 ns, and a X^2 of 170. Compare Figure 1 for 100% 10 ns vs 100% 4 ns.

number, (b) poorly spaced, or (c) inappropriate frequencies are chosen. Similarly, if the two monoexponential lifetimes are too close, the expected phase differences and demodulation ratios will be rather close to the precision of the instrument, and the result will be poorly defined (see Figure 3 and Theory). We note, however, that the lifetimes of Sulforhodamine 101 and Rhodamine 6G, which differ by less than 10%, were accurately recovered by our method (Table I). Monoexponentials that differ very widely in lifetime behave as if the shorter were a scatterer; i.e., at any frequency at least one of the monoexponentials has a near zero or 90° phase shift relative to the excitation, and it is therefore necessary to measure over an adequate frequency range.

In the foregoing we have assumed that both standards are monoexponentials, and experience and other workers have shown this to be a good assumption for the compounds listed in Table I. However, our method also enables us to test this assumption for compounds not previously known to be monoexponential. In particular, Figure 7 shows simulated phase differences and demodulation ratios for a 4-ns monoexponential and a second "standard" that is not quite a pure monoexponential (e.g., 95% 10 ns, 5% 1 ns). The lines in the figure indicate the best two monoexponential fit to the data, in this case values of 12.64 and 6.43 ns and X^2 of 170 (compare Figure 1). The X^2 for the impurity-containing pair is very high, and the differences between the simulated values and those calculated from the best fit (the residuals) are clearly nonrandom and more than 10-fold larger than the precision of the instrument. It is clear, therefore, that our method can detect impurities as small as 5% in a "pure" compound. For this simulation, we have assumed no instrumental noise and equal precision even at high frequencies, which are not found in practice. However, we do not feel that either of these factors affects the validity of our simulation or conclusions. Impurities with lifetimes similar to the major component are much less detectable in this scenario. In particular, the same simulation but with a 10% impurity having a 5-ns lifetime in the 10-ns "standard" (e.g., 90% 10 ns, 10% 5 ns vs 4 ns) resulted in only a 6-fold increase in X^2 over the data in Figure 1. Therefore, while we initially assume both members of a pair are monoexponential, this assumption can be fairly well tested.

Accuracy of the Method. It was stressed that the accuracy of a standard can impose an upper limit on the accuracy of a lifetime measurement, as has been shown by Rayner et al. (2). For the reasons stated above, our method should be as least as accurate as using a scatterer, but an independent test is still useful. We performed a quenching experiment that

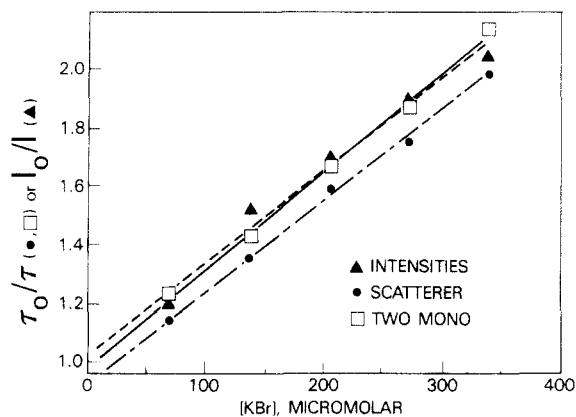


Figure 8. Stern-Volmer plot of KBr quenching of SPA in water. Data and the best fit linear regression lines are shown for intensities (\blacktriangle , ---), lifetimes by the two monoexponential algorithm (\square , —), and lifetimes using a scatterer (\bullet , ---).

is a sensitive test of the accuracy of our method (6). The basis of the test is that for a fluorophore/quencher system that exhibits purely collisional quenching and a fully accessible fluorophore, Stern-Volmer plots of lifetime will be linear. Thus the correlation coefficient of the best fit linear least-squares line will reflect the accuracy of the lifetime determinations. We chose to study the quenching of *N*-(3-sulfo-propyl)acridinium by potassium bromide in distilled water (10). This fluorophore/quencher combination was chosen because the efficient quenching by the bromide and the long lifetime of the probe permit the use of micromolar concentrations of quencher, minimizing ionic strength effects. Furthermore, the zwitterionic character of the probe minimizes ionic attractions for the quencher, and thus static quenching. Finally, the probe is known to exhibit a linear intensity Stern-Volmer plot up into the millimolar range of bromide concentration, and thus it should also be linear at lower concentrations (10). Thus we measured the lifetime of quenched SPA samples versus a scatterer and versus unquenched SPA using our method; the data are shown in Figure 8. The Stern-Volmer plots in Figure 8 of intensity and lifetime are in fact practically identical, showing that the quenching process is collisional. The correlation coefficients of the intensity, lifetime using a scatterer, and lifetime lines using our method were 0.9945, 0.9974, and 0.9995, respectively. According to this criterion, our method is more accurate than that of a scatterer.

Methods have been described in the literature for time-correlated single photon counting data which are roughly similar to our frequency domain method (5, 16, 17). Zuker et al (17), describe a "delta function convolution method" where no value need be assumed for the reference compound, but there is an absolute requirement that it be monoexponential; likely it could be adapted to compare two monoexponential standards as we have done. Wijnaendts van Resandt et al (16), describe a double beam pulse instrument that employs an external reference material and light path in a manner reminiscent of that of Lakowicz and Weber (18). Finally, Castelli (5) describes a method of standard lifetime determination based on analysis of several data sets to generate a best fit decay for a putative standard.

While it is evidently difficult to compare these impulse response methods with our frequency domain method, our

approach has some useful features. Our method explicitly tests the assumption that the standards are monoexponential, using objective criteria. Also, it is simple, requiring neither many data sets nor instrument modification. It is evidently a completely general technique, useful in any modulation frequency range or spectral band.

Finally, it is important to note that a more accurate means of determining fluorescence standard lifetimes could have substantial utility. In particular, the ability to resolve multicomponent decays, especially short ones, depends critically on the accuracy of multifrequency phase and modulation data and thus standard lifetimes (8, 11). Moreover, more detailed molecular information could be derived from experiments involving lifetimes, e.g., those using quenching or energy transfer as molecular probes. Experiments are under way in our laboratories applying this new method to complex biochemical systems.

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Registry No. Me₂POPOP, 36812-21-2; MSQ, 83907-40-8; SPA, 83907-41-9; Rhodamine 6G, 989-38-8; Rose Bengal, 11121-48-5; 9-cyanoanthracene, 1210-12-4; anthracene, 120-12-7; Sulforhodamine 101, 60311-02-6; Rubrene, 517-51-1; glycogen, 9005-79-2; potassium bromide, 7758-02-3.

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