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Fluorescence Lifetime Reference Standards for the Range 0.189 to 115 Nanoseconds

RAYMOND F. CHEN

Laboratory of Technical Development, National Heart and Lung Institute, Bethesda, Maryland 20014

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A review of the modern literature reveals that the values for quinine fluorescence lifetime are in good agreement, the mean value being 18.91 ± 0.56 nsec. By virtue of some very unusual properties, quinine appears suitable for use as a lifetime reference standard for any value from 0.189 to 18.9 nsec, with an expected accuracy of ±3% throughout this range. Cl^-, not normally considered a quenching agent, quenches quinine emission at diffusion-controlled rates. The Stern–Volmer plot was unique in that the strict linearity, indicating pure collisional quenching, was maintained even when fluorescence was >99% quenched. Thus, solutions of quinine–NaCl can be made up having lifetimes known to great accuracy. Similarly, γ-pyrenebutyrate solutions containing KI are suitable standards for the range up to 115 nsec. The compositions of such solutions have been calculated and tabulated. It is argued that the lifetimes of these solutions are at least as reliable as any of the hundreds of lifetimes which have been reported in the literature. Several important applications of such lifetime standards are discussed.

Advances in instrumentation over the last few years have made the measurement of fluorescence lifetime a practical procedure in many laboratories. The lifetime τ is one of the basic parameters of fluorescence, and its importance and measurement have been reviewed by Birks and Munro (1). Modern instrumentation for measuring fluorescence lifetime are generally characterized by excellent *precision* and time resolution, yet the literature abounds with discrepancies, so that the *accuracy* of a given instrument may be in question. Reliable fluorescence lifetime standards would be useful to test the performance of an apparatus and facilitate the determination of decay times of unknowns. Although many lifetime values have been reported, there is no agreement on what is the most suitable compound for a standard. Many of the reported lifetimes were determined for substances dissolved in specific organic solvents which must first be deaerated. Such values have little usefulness for

studies in the biological area, which deals primarily with aqueous solutions.

In this communication, literature values of the lifetime of quinine in 0.1 N sulfuric acid are reviewed and found to be in good agreement. Further, we demonstrate an unusual quenching of quinine by chloride ion which proceeds at diffusion-controlled rats and gives a Stern–Volmer plot which is linear to very high degrees of quenching. On the basis of these findings, it is proposed that quinine–NaCl solutions can be made up to yield any desired lifetime (± 3%) from 0.189 to 18.9 nsec. Similarly, γ-pyrenebutyrate–KI solutions having lifetimes up to 115 (± 3%) nsec are also good standards. Finally, the need for such standards in making "absolute" measurements of lifetime is stressed, along with other possible applications.

MATERIALS AND METHODS

Crystalline quinine bisulfate (Eastman, Sigma, or Merck) was used in these studies. No difference in results could be found with the different samples or with samples recrystallized from water. These findings are in harmony with the experience of Fletcher (2). Eastman γ-pyrenebutyric acid was recrystallized from ethanol–water mixtures. Additional support for the purity of the compounds was the finding that their emission spectra were independent of the exciting wavelength over a range from 250 nm to the excitation maxima.

Lifetimes were measured on a TRW Instruments (El Segundo, CA) decay time apparatus (3). A N_2 spark lamp and Corning CS 7-54 and 3-74 primary and secondary filters were used. Some values and decays were also examined with an ORTEC 9200 nanosecond single-photon spectrometer.

Static fluorescence intensity measurements were obtained with an Aminco–Bowman spectrophotofluorometer. All measurements were made at 23°, and the solutions were not deaerated. The data for the Stern–Volmer plots were obtained with 1-cm path length cells and carefully corrected for solvent blank, which was quite significant for quinine solutions quenched by more than 98%. Excitation and emission bandwidths were 24 nm.

RESULTS

Table 1 summarizes literature values for the fluorescence lifetime of quinine in 0.1 N H_2SO_4. Most of the values were originally reported for 1.0 N H_2SO_4 solutions, but these have been corrected by multiplying by 0.94 to give the lifetime in the more dilute acid. The rationale for this

TABLE 1
Reported Values of Fluorescence Lifetime of Quinine in 0.1 N H_2SO_4

Lifetime[a] τ (nsec)	Reference
18.05[b]	Berlman (22)
18.24[b]	Ware and Baldwin (23)
18.89[b]	Birks and Dyson (24)
19.27[b]	Metcalf (25)
19.0	Chen *et al.* (3, 7)
19.4	Yguerabide (19)
19.5	Weber (26)

[a] Mean = 18.91 nsec. Standard deviation = 0.56 nsec.
[b] Obtained by multiplying by 0.94 the value reported for quinine in 1 N H_2SO_4.

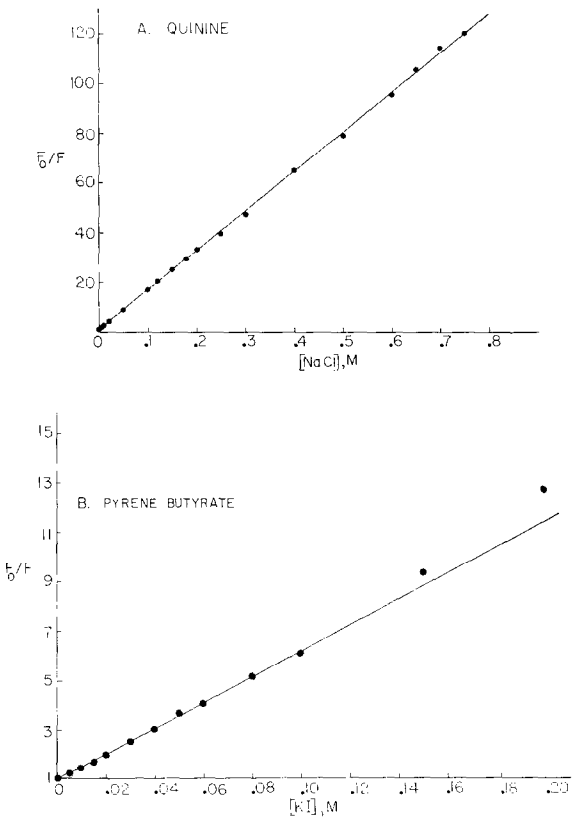

FIG. 1. Stern–Volmer quenching plots for quinine (A) and γ-pyrenebutyrate (B). F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively. Excitation and emission wavelengths were 350 and 450 nm for A and 335 and 390 nm for B. See Tables 2 and 3 for solution makeup.

correction is that it is known that the quantum yield (4–7) as well as the fluorescence lifetime (7) is significantly dependent on the sulfuric acid strength, being 6% higher in 1.0 N rather than 0.1 N H_2SO_4. The mean of the values in Table 1 is 18.91 nsec with a standard deviation of 0.56, or 3%, and a standard error of 0.21. Because of this rather good agreement, the lifetime of quinine may be considered to be well-established.

Chloride ion has long been known to quench quinine fluorescence (8). Figure 1A shows that the quenching follows an extremely linear Stern–Volmer plot. NaCl, $CaCl_2$, KCl, and NH_4Cl gave the same curves, so it is likely that the cation had no effect. The strict linearity was retained to an F_0/F value of at least 100, a finding strongly indicating that the quenching is purely dynamic (collisional) at all concentrations of chloride ion. To this author's knowledge, no other condensed-phase system has

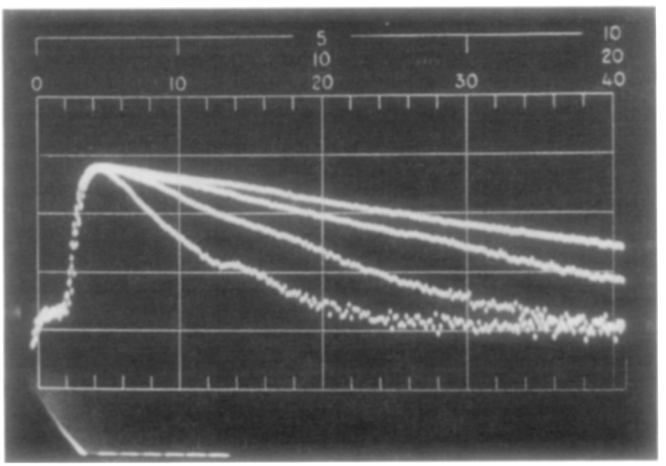

FIG. 2. Direct evidence for shortening of the decay time of quinine fluorescence by NaCl. Shown is the cathode ray tube display of the memory contents of the multichannel analyzer component of the ORTEC 9200 nanosecond single-photon spectrometer. Samples of quinine in 0.1 N H_2SO_4 with and without NaCl were excited repetitively with an air spark lamp, and the emitted single photons were counted and recounted in separate channels corresponding to different times after the initiation of the excitation. Primary and secondary filters were Corning 7-54 and Wratten No. 8. The plot coordinates are log counts vs channel number (time). The vertical divisions are log units, and the large horizontal divisions represent 21.45 nsec. Some irregularity is injected due to a slight imperfection in the log display, but most of the nonlinearity (especially notable in the fastest decay) is due to the convolution with the lamp pulse shape. The solutions had lifetimes of 18.9, 13.0, 7.00, and 3.00 nsec, and were made up as indicated in Table 2. The decay kinetics of each sample were recorded on 256 channels, the peak channel of which contained 20,000 counts; the curves were then displayed together.

TABLE 2
Composition of Quinine Solutions for Lifetime Standards[a]

Lifetime (nsec)	x (ml)	y (ml)
18.9[b]	0.00	8.90
18.5	0.013	8.89
18.0	0.031	8.87
17.5	0.049	8.85
17.0	0.068	8.83
16.5	0.090	8.81
16.0	0.112	8.79
15.5	0.136	8.76
15.0	0.161	8.74
14.5	0.188	8.71
14.0	0.217	8.68
13.5	0.248	8.65
13.0	0.281	8.62
12.5	0.318	8.58
12.0	0.357	8.54
11.5	0.399	8.50
11.0	0.445	8.46
10.5	0.496	8.40
10.0	0.552	8.35
9.50	0.614	9.29
9.00	0.683	8.22
8.50	0.759	8.14
8.00	0.845	8.06
7.50	0.944	7.96
7.00	1.05	7.85
6.50	1.18	7.72
6.00	1.33	7.57
5.50	1.51	7.39
5.00	1.73	7.17
4.50	1.99	6.91
4.00	2.31	6.59
3.50	2.73	6.17
3.00	3.29	5.61
2.50	4.07	4.83
2.00	5.25	3.65
1.50	7.20	1.70
1.00	1.11[c]	7.79
0.50	2.29[c]	6.61
0.189	6.15[c]	1.75

[a] Stock solutions: quinine bisulfate (6), 0.056 g dissolved in 100 ml H_2O; NaCl, 0.1 and 1.0 M solutions; 1.0 N H_2SO_4. Each tube contains 1.0 ml 1 N H_2SO_4, 0.10 ml quinine, x ml of 0.1 M NaCl, and y ($= 8.90 - x$) ml H_2O.

[b] Mean of values listed in Table 1. All other lifetimes are those calculated to be given by the amount of NaCl present. See text for details.

[c] Use 1.0 M NaCl.

been reported which has maintained Stern–Volmer linearity to such a high degree of quenching.

Decay time measurements with either a TRW Instruments decay time apparatus or an ORTEC 9200 nanosecond spectrometer showed direct proportionality of lifetime and quantum yield. Figure 2 is direct evidence showing the increased rate of deactivation in the presence of NaCl. The Stern–Volmer equation (9) is given by $F_0/F = 1 + K_{SV}[Q]$, where F_0 and F are the fluorescence quantum yields in the absence and presence of quencher (Q), respectively, and K_{SV} is the Stern–Volmer constant. K_{SV}, in turn, is equal to $k_q\tau_0$, where k_q is the bimolecular quenching rate constant and τ_0 is the lifetime in the absence of added quencher. K_{SV} was found to be 161 M^{-1}, so $k_q = 8.51 \times 10^9$ $M^{-1}sec^{-1}$, indicating that the quenching rate is essentially diffusion limited. Since $\tau_0/\tau = F_0/F$, the concentration of NaCl required for any desired lifetime can be calculated from the relation $\tau_0 = \tau(1 + K_{SV}[Q]) = \tau(1 + 161[NaCl])$. The composition of a series of quinine–NaCl solutions covering a range of fluorescence lifetimes from 18.9 to 0.189 nsec was calculated on an Olivetti programmable calculator and listed in Table 2. The determination of the Stern–Volmer constant was quite precise, the standard deviation for six measurements being 0.6%. Although some of the points shown in Fig. 1A are slightly off the linear regression line, these points represent F_0/F values greater than about 30 where blank corrections were necessary. At lower values of F_0/F, all the points fall on a straight line.

The solutions are stable for at least 6 months if kept in stoppered tubes in the dark. Small stoppered vials which fit directly into the decay time fluorometer and never need be opened have been found quite convenient. Quinine fluorescence in sulfuric acid solutions has a relatively small temperature coefficient (7). The studies reported here were done at 23°.

A disadvantage of these standard solutions is the low quantum yield of the standards having short lifetimes. The quantum yield of quinine in the absence of NaCl in 0.1 N H_2SO_4 is 0.51 (5–7,10) and would be reduced by 100-fold when the lifetime is reduced to 0.189 nsec. However, the quantum efficiency is still high enough to render the solutions useful with modern instruments. Furthermore, to offset the low yields, higher quinine concentrations could be used.

Because quinine in 0.1 N H_2SO_4 is a suitable standard only to 18.9 nsec, another substance soluble in water was sought for longer lifetimes. γ-Pyrenebutyric acid, introduced by Knopp and Weber (11) for labeling of proteins, was the most suitable compound found. The substance is sparingly soluble in water, yet enough material dissolved to give a strong fluorescence signal. KI was found to be a convenient quencher, and the

TABLE 3
Composition of γ-Pyrenebutyrate Solutions for Lifetime Standards[a]

Lifetime τ (nsec)	x (ml)	y (ml)
115[b]	0.000	8.00
114	0.003	8.00
113	0.006	7.99
112	0.010	7.99
111	0.013	7.99
110	0.017	7.98
109	0.021	7.98
108	0.024	7.98
107	0.028	7.97
106	0.032	7.97
105	0.036	7.96
104	0.040	7.96
103	0.044	7.96
102	0.048	7.95
101	0.053	7.95
100	0.057	7.94
99	0.061	7.94
98	0.066	7.93
97	0.071	7.93
96	0.075	7.93
95	0.080	7.92
94	0.085	7.92
93	0.090	7.91
92	0.096	7.90
91	0.101	7.90
90	0.106	7.89
89	0.112	7.89
88	0.117	7.88
87	0.123	7.88
86	0.129	7.87
85	0.135	7.87
84	0.141	7.86
83	0.148	7.85
82	0.154	7.85
81	0.161	7.84
80	0.168	7.83
79	0.175	7.83
78	0.182	7.82
77	0.189	7.81
76	0.197	7.80
75	0.205	7.80
74	0.213	7.79
73	0.221	7.78
72	0.229	7.77
71	0.238	7.76

TABLE 3 (*Continued*)

Lifetime τ (nsec)	x (ml)	y (ml)
70	0.246	7.75
69	0.256	7.74
68	0.265	7.74
67	0.275	7.73
66	0.285	7.72
65	0.295	7.71
64	0.306	7.69
63	0.317	7.68
62	0.328	7.67
61	0.340	7.66
60	0.352	7.65
59	0.365	7.64
58	0.377	7.62
57	0.391	7.61
56	0.405	7.60
55	0.419	7.58
54	0.434	7.57
53	0.449	7.55
52	0.465	7.54
51	0.482	7.52
50	0.500	7.50
49	0.517	7.48
48	0.536	7.46
47	0.556	7.44
46	0.576	7.42
45	0.598	7.40
44	0.620	7.38
43	0.643	7.36
42	0.668	7.33
41	0.693	7.31
40	0.721	7.28
39	0.749	7.25
38	0.779	7.22
37	0.810	7.19
36	0.843	7.16
35	0.878	7.12
34	0.916	7.08
33	0.955	7.05
32	0.997	7.00
31	1.041	6.96
30	1.089	6.91
29	1.140	6.86
28	1.195	6.81
27	1.253	6.75
26	1.316	6.68
25	1.384	6.62

TABLE 3 (*Continued*)

Lifetime τ (nsec)	x (ml)	y (ml)
24	1.458	6.54
23	1.538	6.46
22	1.625	6.38
21	1.721	6.28
20	1.826	6.17
19	1.943	6.06
18	2.072	5.93

[a] Stock solutions: saturated γ-pyrenebutyrate (9): shake 10 mg γ-pyrenebutyric acid with 20 ml 0.01 M KOH and filter; 0.1 M Tris–Cl^- buffer, pH 8.0; 0.5 M KI solution: 4.15 g KI and 0.0013 g sodium thiosulfate made up to 50.0 ml. Each tube contains 1.0 ml γ-pyrenebutyrate solution, 1.0 ml Tris–Cl^- buffer, x ml KI solution, and y ($= 8.00 - x$) ml H_2O.

[b] Measured lifetime. All other lifetime values are those calculated to be given by the amount of KI present, based on the Stern–Volmer relation.

Stern–Volmer relation was found to be followed up to a value of F_0/F somewhat above 6 (Fig. 1B). The lifetime of γ-pyrenebutyrate was found to be 115 nsec in the absence of quencher, so solutions containing KI will serve as standards down to about 18 nsec.

The quenching curve for γ-pyrenebutyrate and KI gives $K_{SV} = 51.9\ M^{-1}$. With $\tau = 115$ nsec, k_q is calculated to be $4.51 \times 10^8\ M^{-1}sec^{-1}$. Therefore, the concentration of KI needed to obtain a solution with a given lifetime may be calculated from the relation $115 = \tau\ (1 + 51.9\ [KI])$. Table 3 gives the composition of standard solutions containing γ-pyrenebutyrate and KI which have lifetimes covering the range 18 to 115 nsec.

Compared with quinine solutions, γ-pyrenebutyrate solutions are less convenient, since both I^- and the fluorescent compound are rather unstable. Thiosulfate is included to retard I_3^- formation, and the solutions can be stabilized to some extent by deaeration. However, it is known that γ-pyrenebutyrate is easily photolyzed (12), and so the standard solutions should be made up on the day of their use.

Another deficiency of the γ-pyrenebutyrate–KI system is the absence of a large number of reported values for the lifetime of γ-pyrenebutyrate in water. Knopp and Weber (13) attached the γ-pyrenebutyrate chromophore to serum albumin and a macroglobulin and measured lifetimes ranging from 77 to 119 nsec for the former and 100 to 137 nsec for the latter, depending on the preparation. Rawitch *et al.* (14) made conjugates of thyroglobulin containing γ-pyrenebutyrate; τ values of two samples were 90 and 125 nsec. Jonas' (15) preparations of γ-pyrenebutyrate conjugates of serum high-density lipoprotein had lifetimes of 62–70 nsec.

Spencer *et al.* (12) found the lifetime of free γ-pyrenebutyric acid in 1,2-propanediol to be 195 nsec. The lifetime of this substance, therefore, clearly varies widely depending on its milieu. Vaughan and Weber (16) have studied the effect of O_2 quenching on the fluorescence lifetime of γ-pyrenebutyric acid both free in solution and bound to macromolecules. Their value of the lifetime of the free compound in pH 8.0 phosphate buffer at 25°, given as a point in their Fig. 5, appears to correspond to 113 ± 10 nsec at an oxygen tension of 150 mm Hg. The conditions correspond closely to those used in this study, and the lifetime obtained agrees closely with the value reported here, 115 nsec for an air-saturated solution. The value was obtained with the TRW fluorescence decay time apparatus; the standard deviation from the mean of replicate measurements was 3%. Pyrenebutyrate and quinine solutions with calculated lifetimes of 18.0 nsec appeared to have identical decay curves when examined with the ORTEC spectrometer.

DISCUSSION

A major thesis of this communication is that the lifetimes of the quinine solutions described here are known with better accuracy than those of any other solutions described in the literature. The validity of this statement is not immediately obvious since the lifetimes of hundreds of materials have now been reported. However, perusal of the extensive tables of lifetimes in the review by Birks and Munro (1), for example, shows no other substance whose lifetime has been measured by so many laboratories with such good agreement as in the case of quinine. This is especially true of substances of rather short lifetime, e.g., 2 or 3 nsec, where *precision*, not to mention accuracy, has rarely been claimed to be as good as 3%. In contrast to this situation, there is no reason to believe that the calculated lifetime of, say, 0.189 nsec of the appropriate quinine–NaCl solution is any less *accurate* than 3%.

The need for reliable fluorescence standards to check and calibrate a new instrument and to detect any systematic errors is obvious. However, one might ask why fluorescence standards for lifetime measurements are needed, since most lifetime methods are "absolute" in the sense that they do not seem to require a reference standard. The answer to this question requires appreciation of the fact that characterization of lifetime instruments as "absolute" is somewhat deceptive, in that such measurements are not, in fact, "direct." Each device described for measuring decay times of fluorescence is dependent on a standard, which usually is a standard of zero lifetime such as a scattering or reflecting sample. Such a $\tau = 0$ standard is used in both phase fluorometers and pulsed systems. Possibly one of the major causes of error in lifetime

determinations is due to the assumption that once an instrument has been calibrated to read zero with such a sample, it will behave linearly and yield the correct value with samples of even very long lifetimes. The situation is analogous to the measurement of pH: with a perfect pH meter, it would make no difference what standard was used; but no careful chemist would standardize at pH 2 when dealing with a sample with a pH near 10. Similarly, it would also be preferable to standardize a lifetime apparatus with a standard of a lifetime near that of the unknown.

At present, the TRW fluorescence decay time apparatus is probably the most widely used instrument of its type, and it too requires a standard. While a scattering solution was recommended as such a standard (3), a fluorescence standard would probably enhance the accuracy of the measurements. Curve matching can be performed with the decay time dial set to the lifetime of the standard solution.

In many decay time instruments using repetitive-pulsed excitation, the fluorescence vs time curve is a complicated function representing the convolution of the light pulse shape with the fluorescence decay curve which would have been observed with a true delta-function impulse. In order to obtain the decay curve, deconvolution by digital computer can be performed [e.g., (17–19)] if one knows the lamp pulse shape. A simplified variation of the technique was recently described by Lytle (20). In his method, the observed fluorescence curve, plotted by an X-Y recorder, is compared with transparencies created by computer convolution of the lamp impulse function and exponential decay curves representing different lifetimes. With fluorescence lifetime standards, one can essentially generate such convolution functions experimentally rather than by computer. To determine lifetimes, one needs only to match the unknown's curve with that of the series of standards. This procedure would minimize errors caused by random variations in the lamp pulse shape. Naturally, comparison of unknown and standard curves need not be done by the transparency method (20). Subjective errors could be avoided by testing the fit of curves by computer, for instance.

Detailed analysis of the decay kinetics of quinine (19) and excitation of γ-pyrenebutyric acid with light modulated at different frequencies in a phase fluorometer (12) show that these compounds decay according to a simple first-order exponential process. Whereas fluorescence decay is due to more than one component, the decay curve will deviate from a simple exponential. Much effort has been expended in attempting mathematical procedures to resolve the complex decays [e.g., (19,21)]. Since quenching by Cl^- is quite uncommon, model solutions could be made up of a test substance and quinine–NaCl to serve as the second com-

ponent, whose decay time would be varied depending on the amount of Cl^- added. The decay curves from such model solutions could be compared with experimental curves to detect multicomponent decays or to test mathematical procedures for resolution of multiple component decays.

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