ANALYSIS OF NONEXPONENTIAL FLUORESCENCE DECAY DATA BY A METHOD OF MOMENTS

T. N. SOLIE, E. W. SMALL, AND I. ISENBERG, *Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331 U.S.A.*

ABSTRACT A method of moments is presented for the analysis of convoluted fluorescence decay data when the impulse response function is given by $f(t) = \alpha \exp(-At - Bt^{1/2})$. Examples of this method are given using both simulated and measured fluorescence decays. It is also shown that this method, used with moment index displacement, will correct for light-scatter leakage, zero-point time shifts, and slow lamp drift.

INTRODUCTION

Several methods have been presented for the analysis of fluorescence decay data that can be represented as a sum of exponentials (1–17). While such a representation covers a wide variety of experimental situations, other functional forms also arise. In this paper we present a method of moments for analyzing fluorescence decay data when the impulse response function has the form

$$f(t) = \alpha \exp(-At - Bt^{1/2}). \tag{1}$$

This form arises in the study of radiationless singlet-singlet energy transfer (18–21), and in the study of diffusion-controlled quenching of fluorescence (22, 23). For radiationless energy transfer (20), A is the reciprocal of the donor excited-state lifetime in the absence of acceptors, and B is a function of the nature, separation, and orientation of the donor-acceptor pair of chromophores. For diffusion-controlled quenching of fluorescence the A parameter is composed of two parts: the reciprocal of the excited-state lifetime in the absence of quencher, and a term that is a function of collision radii and diffusion coefficients of the diffusing species and the concentration of the quencher. The B parameter is proportional to the product of the concentration of the quencher, the square of an encounter distance, and the square root of the relative diffusion coefficient (23).

The method of moments, with moment index displacement (MD), corrects for light-scatter leakage, zero-point shifts, and slow lamp drift when the decay is a sum of exponentials (13, 24, 25). In this paper we show that these corrections are also made for decays of the form of Eq. 1.

THEORY

The observed fluorescence, $F(t)$, is related to the excitation, $E(t)$, by the convolution:

$$F(t) = \int_0^t E(t-u) f(u) \mathrm{d}u, \tag{2a}$$

where $f(t)$ is the impulse response function.

Dr. Solie's present address is Departments of Physiology and Biophysics and of Chemistry, Colorado State University, Fort Collins, Colo. 80523

If light scatter contaminates the fluorescence, then the observed emission will be given by

$$F(t) = \int_0^t E(t - u) f(u) \mathrm{d}u + \xi E(t), \tag{2b}$$

where ξ is the light-scatter leakage coefficient. For

$$f(t) = \alpha \exp(-At - B\sqrt{t}) \tag{3a}$$

$$= \alpha \exp(-t/\tau_1 - \sqrt{t/\tau_2}), \tag{3b}$$

the problem is to obtain the parameters A and B from the fluorescence decay data. We call τ_1 and τ_2 the characteristic lifetimes.

The kth moments of F, E, and f are

$$\mu_k = \int_0^\infty t^k F(t) \mathrm{d}t \tag{4}$$

$$m_k = \int_0^\infty t^k E(t) \mathrm{d}t \tag{5}$$

$$\phi_k = \int_0^\infty t^k f(t) \mathrm{d}t. \tag{6}$$

On substitution of $x = t^{1/2}$, followed by $y = (2A)^{1/2}x + B/(2A)^{1/2}$, it can be shown that

$$\phi_k = \frac{-\alpha}{2^k A^{k+1}} K(c)^{(2k+1)}, \tag{7}$$

where

$$c = B/(2A)^{1/2} \tag{8}$$

and

$$K(c)^{(2k+1)} = -\exp(c^2/2) \int_c^\infty (y - c)^{2k+1} \exp(-y^2/2) \mathrm{d}y. \tag{9}$$

The functions $K^{(j)}$ have been tabulated by Sheppard (26) for $0 \leq j \leq 16$.

From Eq. 7 we have

$$\phi_0 = (-\alpha/A) K^{(1)}(c) \tag{10a}$$

$$\phi_1 = (-\alpha/2A^2) K^{(3)}(c) \tag{10b}$$

$$\phi_2 = (-\alpha/4A^3) K^{(5)}(c) \tag{10c}$$

$$\phi_3 = (-\alpha/8A^4) K^{(7)}(c) \tag{10d}$$

$$\phi_4 = (-\alpha/16A^5) K^{(9)}(c). \tag{10e}$$

Using the first three moments we obtain

$$\frac{\phi_0 \phi_2}{\phi_1^2} = \frac{K^{(1)} K^{(5)}}{[K^{(3)}]^2}. \tag{11}$$

With the next three moments, corresponding to an MD of 1,

$$\frac{\phi_1\phi_3}{\phi_2^2} = \frac{K^{(3)}K^{(7)}}{[K^{(5)}]^2}. \tag{12}$$

For an MD of 2, we obtain

$$\frac{\phi_2\phi_4}{\phi_3^2} = \frac{K^{(5)}K^{(9)}}{[K^{(7)}]^2}. \tag{13}$$

From Sheppard's tables (26), Eqs. 11–13 can be tabulated as functions of c. Given any of the ratios of moments, c may be obtained from the tabulations. The parameters α and A may then be obtained from Eqs. 10 and the parameter B from Eq. 8.

To determine the ϕ_k's from the experimental data for $F(t)$ and $E(t)$ we use the moments μ_k and m_k. It can be shown that (4):

$$\begin{aligned}
\mu_0 &= \phi_0 m_0 \\
\mu_1 &= \phi_0 m_1 + \phi_1 m_0 \\
\mu_2 &= \phi_0 m_2 + 2\phi_1 m_1 + \phi_2 m_0 \\
&\vdots \\
\mu_k &= \sum_{i=0}^{k} \frac{k!}{(k-i)!i!}\phi_i m_{k-i}.
\end{aligned} \tag{14}$$

MATERIALS AND METHODS

Chemicals

The carbon tetrabromide and 1,2 propanediol were obtained from Aldrich Chemical Company, Inc., (Milwaukee, Wis.) and J. T. Baker Laboratory Chemicals (Phillipsburg, N.J.) respectively. They were used without further purification. The 1,2 benzanthracene was obtained from Sigma Chemical Co. (St. Louis, Mo.). It was recrystallized from ethanol and vacuum-sublimed before use.

Sample Preparation

Solutions were prepared with minimum exposure to light, stored in the dark, and used within 48 h of preparation. Prepurified dry nitrogen was bubbled through the solutions to remove oxygen. The degassing was repeated until a constant lifetime was obtained for the 1,2 benzanthracene in the absence of carbon tetrabromide, or until the recovered A and B parameters showed no change with additional degassing. All experiments were done at 20.0 ± 0.1°C.

Absorption, uncorrected excitation, and emission spectra were taken with a Cary 14 spectrophotometer, (Cary Instruments, Fairfield, N.J.) and a Hitachi MPF-2A fluorometer (Hitachi Ltd., Tokyo, Japan).

Instrumentation

A modified monophoton fluorometer (25, 27) was used to obtain experimental decays. Modifications of the original instrument have included use of an RCA 31000M photomultiplier (RCA Solid State, Somerville, N.J.) with quartz window and a sample changing system (28) to reduce the effects of lamp drift.

The excitation source was an Ortec 9200 pulser (Ortec Inc., Oak Ridge, Tenn.) operated at ambient pressure with a slow flow of nitrogen. The flash repetition rate was 5–6,000 flashes/s. Photocurrent windowing (27) was used to reject multiple photon events, and ~15% of the flashes led to collected events.

The exciting wavelength was selected with a 337-nm Baird-Atomic narrow bandpass filter (Baird-Atomic, Inc., Bedford, Mass.). The emission was observed through a Corning 3-75 cutoff filter (Corning Glass Works, Science Products Div., Corning, N.Y.).

The decay curves $E(t)$ and $F(t)$ each occupied 512 channels of a Tracor-Northern multichannel analyzer (Tracor Northern, Middleton, Wis.) and were measured at a channel width of 0.123 ns.

Simulations

All computer programs were written in Fortran IV plus for use on a Digital PDP 11/34 minicomputer (Digital Equipment Corp, Marlboro, Mass.) with double precision arithmetic. The programs are interactive to allow the operator to follow the steps in the calculations as they proceed and to intervene when desirable to select options. The main features of the program are summarized in a block diagram in Appendix I. (Instructions are also provided for interested investigators to obtain copies of the program.)

We have found that the use of real E data to obtain a simulated F by convolution often yields data that cannot be analyzed satisfactorily. It is much better to use a smoothed set of E data. This, of course, corresponds to the actual experimental situation: the real lamp flash is smooth, while the collected E data has superimposed fluctuations. By using such a smoothed E (29), and various values for A and B, F was calculated by Simpson's rule. A pseudo-random-noise generator (30) was used to add noise to E and F, with the standard deviation of counts in any channel given as the square root of the number of counts in the channel. A fraction of $E(t)$ could be added to $F(t)$ as light scatter, and a constant added to simulate background counts. The resulting simulated fluorescence thus takes the form

$$F(t) = F_s(t) + N_r[F_s(t)]^{1/2} + \xi E(t) + BKG,$$

where $F_s(t)$ is the simulated convoluted fluorescence at time t, N_r is a coefficient obtained from the random-number generator, ξ is the light-scatter coefficient (25), and BKG is the background. Illustrative examples for various choices of the parameters α, A, and B are presented in the Tables.

Data Analysis

Experimental fluorescence decay data extends over a finite time, T, only, and not to infinity. To obtain the cut-off correction for the moments we have adopted an iterative procedure similar to that used when $f(t)$ is a sum of exponentials (4). However, the presence of the square root of t term in $f(t)$ forces a modification of the procedure.

Eq. 4 may be rewritten as

$$\mu_k = \int_0^T t^k F(t)\, dt + \delta\mu_k \tag{15}$$

where

$$\delta\mu_k = \lim_{T_1 \to \infty} \int_T^{T_1} t^k F(t)\, dt \tag{16}$$

is the cut-off correction. We initiate the iteration process by obtaining estimates for the parameters α, A, and B. (The method of obtaining these initial estimates is discussed in more detail in Appendix I.) These estimates are used to evaluate the convolution, Eq. 2, and thus, by using Eq. 16, to obtain new $\delta\mu_k$'s. These moment corrections are used to find new ϕ_k's, which are then used to find the next estimates for α,

A, and B. This process is repeated until an arbitrarily small change in α and A is obtained on successive loops.

To evaluate $\delta\mu_k$ for a particular set of α, A, and B, we solve the integral equation (Eq. 16) for $T_1 \simeq 2T$, $4T$, . . . as needed to obtain convergence. For the cases considered here, $T_1 = 8T$ or less is sufficient for convergence up to the fifth moment.

The number of iterations required to find a self-consistent set of decay parameters is reduced by using exponential depression (4). With reasonable values of exponential depression, this process rarely requires more than two loops.

Both simulated and experimental data were analyzed with an exponential depression of ~ 0.1 ns^{-1}. MD's of 0, 1, and 2 were used in analysis of the data. The total counts for each set of data is indicated in the appropriate Table.

The correct analysis of data using this method requires an interplay between channel width, exponential depression, and MD. When a proper choice of these conditions has been made, the recovered decay parameters are independent both of exponential depression and further increments of MD.

TABLE I

SUMMARY OF PARAMETERS OBTAINED FOR VARIOUS SIMULATIONS OF NONEXPONENTIAL FLUORESCENCE DECAY DATA HAVING THE FORM OF EQ. 2*

Data set‡	Parameters								
	α			A			B		
	MD = 0	MD = 1	MD = 2	MD = 0	MD = 1	MD = 2	MD = 0	MD = 1	MD = 2
					(ns^{-1})			(ns$^{-1/2}$)	
1	0.01489	0.01496	0.01497	0.04063	0.04023	0.04015	0.09542	0.09834	0.09897
	0.01509	0.01496	0.01510	0.03909	0.03992	0.03932	0.1057	0.09990	0.1051
	0.01501	0.01508	0.01500	0.03998	0.03952	0.03988	0.1003	0.1035	0.1004
Expected values		0.015			0.04			0.1	
2	0.02000	0.02010	0.02017	0.04006	0.03969	0.03945	0.1998	0.2009	0.2045
	0.02005	0.01986	0.02011	0.03968	0.04033	0.03971	0.2019	0.1967	0.2027
	0.02001	0.10996	0.01998	0.04002	0.04016	0.04010	0.2000	0.1989	0.1993
Expected values		0.02			0.04			0.2	
3	0.05004	0.04983	0.05055	0.03994	0.04025	0.03947	0.5006	0.4981	0.5055
	0.05003	0.05039	0.05064	0.03998	0.03936	0.03900	0.5003	0.5049	0.5077
	0.05024	0.04998	0.05028	0.03054	0.04000	0.03961	0.5035	0.5002	0.5035
Expected values		0.05			0.04			0.5	
4	0.07009	0.06896	0.07024	0.1997	0.2024	0.2002	0.2015	0.1874	0.2001
	0.06992	0.06959	0.07007	0.1999	0.2011	0.1999	0.1995	0.1944	0.2006
	0.07020	0.07050	0.07114	0.1996	0.1987	0.1973	0.2028	0.2069	0.2147
Expected values		0.07			0.2			0.2	
5	0.1008	0.1019	0.1031	0.1979	0.1962	0.1950	0.5097	0.5188	0.5273
	0.1003	0.1014	0.1003	0.2005	0.1988	0.2005	0.5004	0.5096	0.5004
	0.1001	0.1003	0.09392	0.2009	0.2005	0.2108	0.4980	0.5000	0.4432
Expected values		0.1			0.2			0.5	
6	0.2015	0.1993	0.1848	0.2022	0.2057	0.2216	0.9993	0.9862	0.9099
	0.2043	0.1983	0.2411	0.1942	0.2020	0.1673	1.025	0.9926	1.175
	0.2010	0.1973	0.2107	0.2015	0.2053	0.1971	0.9992	0.9808	1.032
Expected values		0.2			0.2			1.0	

*For these examples, distortions were not added to the data. The total counts for E was 1.8×10^6, and parameter α was selected to give total counts of F in the range $3.1\text{–}3.5 \times 10^6$. The exponential depression ranged from 0 to 0.25 ns^{-1}. See text for additional details.

‡Six different sets of decay parameters were chosen for these simulations. These values are given as the expected values. For each set of expected values the results of analyzing these separate simulations are shown.

EVALUATION OF THE METHOD

To evaluate the data analysis procedure, we have used both computer-simulated and experimental data. The tests with simulated decay data permit one to see if the method works. More importantly, it enables one to see directly if MD (2, 24, 25) corrects for light-scatter leakage, zero-point errors, and exciting lamp drift. The tests with real experimental data permit one to see if the error corrections are good enough for practical laboratory usage.

Simulated Data

A typical experimental lamp with full width at half maximum of 2.2 ns, a peak maximum of ~70,000 counts, and total counts of 2×10^6 was chosen. Synthetic data was generated as described in the previous section. Random noise was added to both the lamp and the convoluted fluorescence decays in all cases. Light scatter and background could be added to $F(t)$ to find if the method could automatically correct for these frequently encountered experimental distortions of decay data.

Table I gives a summary of decay parameters obtained using MD's of 0, 1, and 2, as well as the expected values for each set of parameters α, A, and B. The alphas were selected to keep the total counts in $F(t)$ approximately equal to 3×10^6 counts. The values for A correspond to characteristic lifetimes of 25 and 5 ns. The corresponding characteristic lifetimes for the B

TABLE II

SUMMARY OF PARAMETERS OBTAINED SHOWING HOW MD CORRECTS FOR LIGHT SCATTER, ZERO-POINT SHIFTS, AND COMBINED EFFECTS OF THESE DISTORTIONS ON THE DATA*

Light-scatter coefficient	Zero-point shift	Parameters								
		α			A			B		
		MD = 0	MD = 1	MD = 2	MD = 0	MD = 1	MD = 2	MD = 0	MD = 1	MD = 2
						(ns^{-1})			($ns^{-1/2}$)	
0	0.20	0.0258	0.0190	0.0190	0.0088	0.0429	0.0426	0.3065	0.1786	0.1797
0	0.15	0.0248	0.0193	0.0193	0.0110	0.0418	0.0417	0.3738	0.1863	0.1797
0	0.10	0.0235	0.0196	0.0195	0.0163	0.0408	0.0414	0.3355	0.1940	0.1898
0	0.05	0.0216	0.0199	0.0197	0.0299	0.0402	0.0409	0.2609	0.1987	0.1936
0	0.00	0.0201	0.0201	0.0201	0.0397	0.0400	0.0398	0.2029	0.2008	0.2025
0	−0.05	0.0189	0.0202	0.0202	0.0471	0.0396	0.0397	0.1561	0.2038	0.2030
0	−0.10	0.0175	0.0204	0.0203	0.0582	0.0394	0.0397	0.0918	0.2055	0.2035
0	−0.15	0.0160	0.0204	0.0204	0.0737	0.0395	0.0398	0.0860	0.2050	0.2032
0	−0.20	‡	0.0204	0.0205	‡	0.0401	0.0400	‡	0.2015	0.2024
0.2	0.00	‡	0.0200	0.0200	‡	0.0400	0.0400	‡	0.2007	0.2006
0.4	0.00	‡	0.0200	0.0200	‡	0.0401	0.0398	‡	0.1994	0.2012
0.4	0.10	‡	‡	0.0189	‡	‡	0.0421	‡	‡	0.1843
0.4	0.05	‡	0.0177	0.0196	‡	0.0487	0.0407	‡	0.1335	0.1952
0.4	−0.05	‡	0.0230	0.0202	‡	0.0288	0.0403	‡	0.2816	0.1980
0.4	−0.10	‡	0.0273	0.0206	‡	0.0127	0.0403	‡	0.3909	0.1988
Expected values			0.02			0.04			0.20	

*A fraction of the lamp $E(t)$ was added to $F(t)$ to simulate light scatter on the fluorescence data. Total counts for $E(t) = 1.8 \times 10^6$ and for $F(t)$, $3\text{–}4 \times 10^6$. Triplicate $F(t)$'s were analyzed as independent decay curves. The exponential depression used ranged from 0.075 to 0.20 ns^{-1}. See text for additional details.

‡No analysis obtained.

TABLE III
SUMMARY OF PARAMETERS OBTAINED SHOWING HOW MD CORRECTS FOR DRIFT AND SHAPE CHANGES OF THE EXCITATION LAMP*

Lamp	E-shift	Parameters								
		α			A			B		
		MD = 0	MD = 1	MD = 2	MD = 0	MD = 1	MD = 2	MD = 0	MD = 1	MD = 2
	(ns)					(ns^{-1})			($ns^{-1/2}$)	
1	0.00	0.0112	0.0121	0.0121	0.0481	0.0420	0.0420	0.169	0.215	0.215
1	0.05	0.0118	0.0119	0.0119	0.0430	0.0426	0.0428	0.207	0.210	0.209
2	0.00	0.0687	0.0589	0.0596	0.0285	0.0423	0.0415	0.307	0.208	0.215
2	0.05	0.0720	0.0580	0.0585	0.0236	0.0431	0.0426	0.341	0.202	0.206
Expected values						0.04			0.02	

*Lamp 1 was collected with the experimental decay curve: 1.21×10^6 total counts with a maximum of 55,380 counts at channel 123. The sample decay had 1.16×10^6 total counts. Lamp 2 was collected independently of the sample decay with general conditions as similar as possible, but with fewer total counts: 2.39×10^5 total counts with maximum of 12,300 at channel 124. The analyses were done with an exponential depression of 0.1 ns^{-1}.

values are 100, 25, 4 and 25, 4, 1 ns, respectively. The exponential depression used ranged from 0 to 0.25 ns^{-1}. Table I illustrates the capability of the method, but there is no systematic distortion in the data. Consequently, any MD is satisfactory.

The ability of MD to correct for zero-point shifts, light scatter, and light scatter plus zero-point shifts is illustrated in Table II. Clearly, if none of these experimental distortions are present, a good analysis is obtained for an MD of 0, 1, and 2. However, with light scatter, and with zero-point shift distortions on the data, a poor analysis, or none at all, is obtained for MD = 0. These distortions are usually corrected by MD = 1.

The effect of similar shifts and shape changes of experimental lamps on the analysis of decay data is shown in Table III. Here the experimental decay curve for 1,2 benzanthracene in the presence of the quencher, carbon tetrabromide, was collected using a sample changer with alternating accumulation of counts for the lamp and the sample. The first two lines show the resulting analysis and the ability of MD to correct for the apparent E-shift of 0.05 ns. The last two lines show results of an analysis of the same sample decay data, with general experimental conditions as similar as possible, but using a lamp collected independently on a different day. As indicated in the footnote to Table III, lamp 2 has fewer total counts and a new position for the maximum. Although the alphas are expected to be different by a factor corresponding to the ratio of total counts in the lamp and cannot be compared directly, the decay parameters A and B are in good agreement. Also, even though an E-shift could not be found that would yield a consistent analysis for MD = 0, incrementing the moment index corrects for the changes in the shape of the lamp on the recovery of the decay parameters.

Experimental Data

The deconvolution method for nonexponential decays was tested on experimental data obtained as outlined in Materials and Methods. The experimental materials and conditions were those of Nemzek and Ware (22). A typical experimental lamp was characterized approximately by a full width at half maximum of 2.5 ns, a peak of 50,000 counts, and total counts of 10^6.

TABLE IV

VALUES OF α, A, AND B OBTAINED FROM FLUORESCENCE DECAY CURVES* OF 1,2 BENZANTHRACENE QUENCHED BY CARBON TETRABROMIDE IN THE SOLVENT 1,2 PROPANEDIOL AT 20°C.

CBr_4 concentration	E-shift	Parameters								
		α			A			B		
		MD = 0	MD = 1	MD = 2	MD = 0	MD = 1	MD = 2	MD = 0	MD = 1	MD = 2
(M)	*(ns)*									
0.056	0.00	0.0066	0.0070	0.0069	0.0344	0.0302	0.0306	0.126	0.159	0.155
	0.07	0.0070	0.0069	0.0068	0.0301	0.0305	0.0314	0.160	0.156	0.148
0.105	0.00	0.0113	0.0121	0.0120	0.0490	0.0439	0.0440	0.173	0.212	0.210
	0.07	0.0120	0.0121	0.0120	0.0436	0.0436	0.0439	0.213	0.213	0.211
0.105	0.00	0.0112	0.0121	0.0121	0.0481	0.0420	0.0420	0.168	0.215	0.215
	0.07	0.0118	0.0119	0.0119	0.0430	0.0426	0.0428	0.207	0.210	0.209
0.105	0.00	0.0116	0.0121	0.0123	0.0486	0.0450	0.0445	0.182	0.210	0.214
	0.05	0.0121	0.0120	0.0121	0.0446	0.0452	0.0449	0.211	0.207	0.202

*The lamp and sample decay curves typically contained total counts in the range 5×10^5 to 1.5×10^6. Excitation was centered at 337 nm, and emission was through a Corning 3-75 cutoff filter (Corning Glass Works, Science Products Div., Corning, N.Y.).

Experiments with degassed samples of the fluorophor, 1,2 benzanthracene, in 1,2 propanediol at 20°C produced an unquenched lifetime of 37.5 ± 0.5 ns, in fair agreement with the reported (interpolated) value of 39 ns (22). These data were analyzed using the method of moments for sums of exponentials (1).

Table IV contains a summary of the decay parameters obtained for 1,2 benzanthracene in 1,2 propanediol quenched by carbon tetrabromide. All of the procedures developed using simulated decay data work for these real experimental data as well.

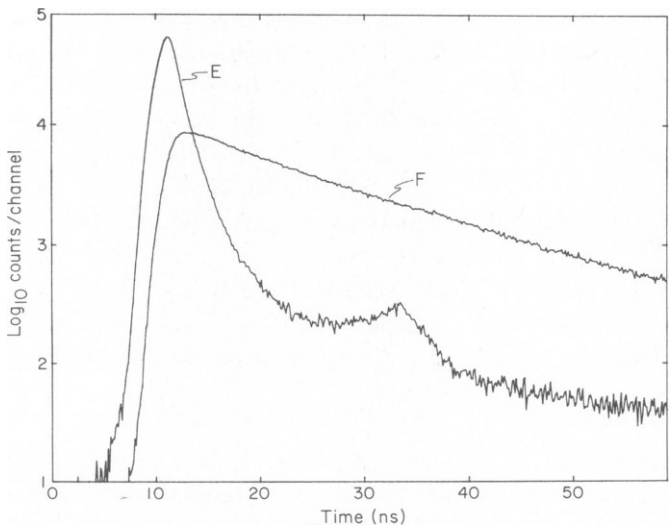

FIGURE 1 Log plots of the lamp flash (E) and the fluorescence decay (F) for 1,2 benzanthracene quenched by 0.105 M carbon tetrabromide.

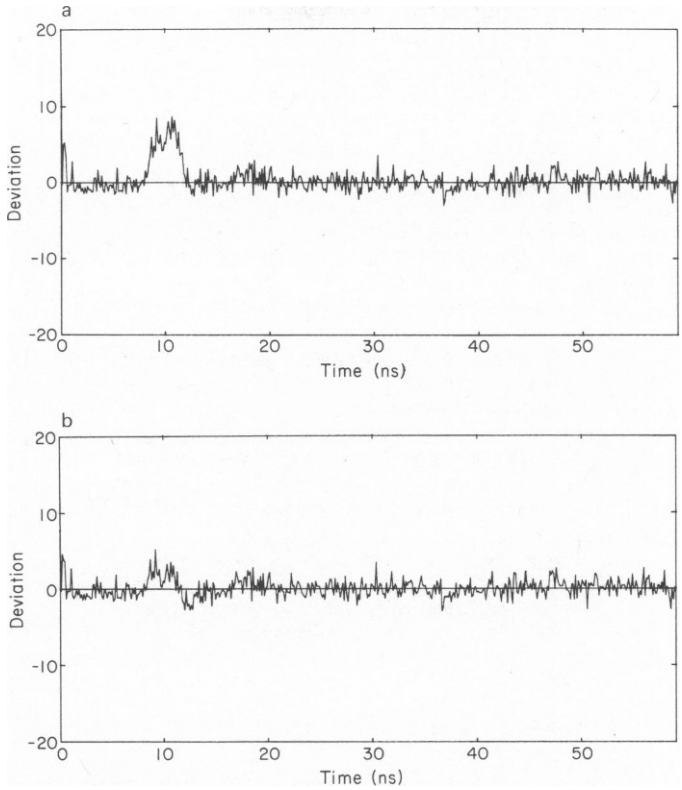

FIGURE 2 Plots of the deviation function for analyses of the data shown in Fig. 1 with (*a*) no *E*-shift applied to the data, and (*b*) after shifting the lamp flash to longer times by 0.1 ns.

Plots of the lamp, E, and the fluorescence decay, F, for the second data set of Table IV are shown in Fig. 1. Fig. 2 shows plots of the deviation function (9) defined as: deviation = $[i_c(k) - i(k)]/i(k)^{1/2}$, where $i(k)$ and $i_c(k)$ are the number of counts in the kth channel of the experimental and calculated curves, respectively. The first deviation plot (Fig. 2 *a*) is from the analysis with MD = 1. The second deviation plot is again for MD = 1, but after a shift has been applied by shifting the time origin used for calculating the moments of $E(t)$ to longer times by 0.07 ns. Note from Table IV that both analyses result in the same answer, although in Fig. 2 *a* the calculated $F(t)$ deviates significantly from the measured curve.

From the results of Nemzek and Ware (22) based on an alternate deconvolution method, approximate expected values for A and B for a quencher concentration of 0.105 M were estimated to be 0.051 ns^{-1} and 0.062 $ns^{-1/2}$, respectively. The value for A obtained in this work differs by 14%; the value for B differs by more than a factor of 3.

DISCUSSION

From the results described for both simulated and experimental decay data, it is clear that MD corrects for consistent errors when the impulse response function has the form of Eq. 1. Usually an MD of order 1 will yield reliable decay parameters. Obtaining consistent sets of

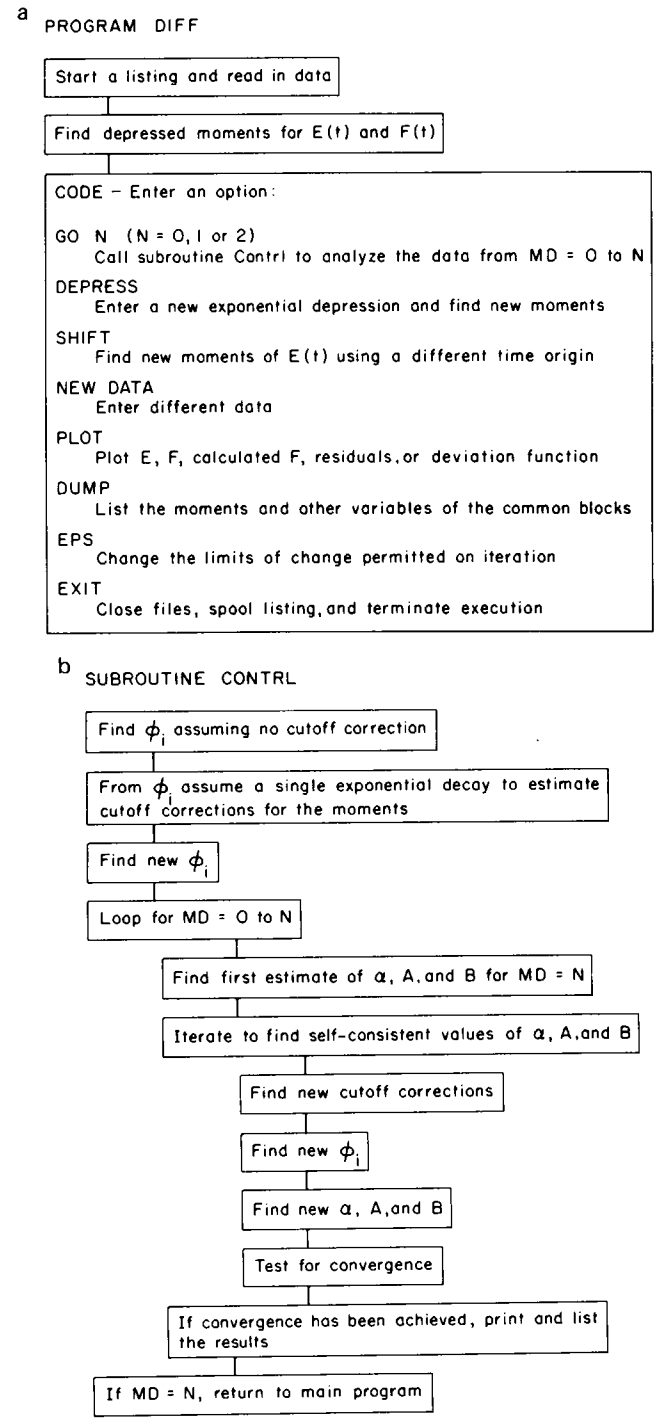

FIGURE 3 Block diagram illustrating the flow of program DIFF. (*a*) After reading in data and finding moments the main program enters the CODE position. CODE acts as a dispatcher, permitting the user to exercise a series of options. (*b*) Subroutine CONTRL directs the analysis of the data.

parameters with a further increase in MD is evidence of the reliability of the recovered parameters.

As demonstrated by using simulated decay data, MD corrects for light scatter on the fluorescence data and minimizes the effects of other instrumental errors such as zero-point shift, and slow changes in lamp profile.

The method of moments, with MD, has been shown previously to be a powerful method in the analysis of fluorescence decay data where the impulse response is a sum of exponentials. In this paper we have shown that the method also works for a specific nonexponential decay. The complete range of validity for corrections by MD is still an open question.

APPENDIX I

Program DIFF is an interactive program written in Fortran IV plus and run on a PDP 11/34 computer (Digital Equipment Corp.) under RSX-11. Figs. 3 *a* and *b* present a block diagram illustrating the flow of this program.

After an initial start-up sequence in which data is read into the program and depressed moments calculated for this data, the program enters the CODE position. This point in the program acts as a dispatcher. The user may enter one of several options, and at the conclusion of the option the program returns again to CODE. If the user types GON, where $N = 0$, 1, or 2, the program calls subroutine CONTRL to analyze the data for MD = 0 up to MD = N.

CONTRL first makes estimates of the parameters α, A, and B. To make these initial estimates, the program assumes that the data is a single exponential decay, uses the uncorrected ϕ_1 and ϕ_2 to solve for a lifetime and preexponential factor, finds cut-off corrections for the moments of F, and uses these single exponential cut-off corrections to find improved ϕ_i. These ϕ_i are in turn used to make the first guess of α, A, and B for MD = 0. Although an alternate sequence could be used to find the initial estimates, by simply assuming the moment corrections are zero, rather than the result of a single exponential decay, such estimates are not as good. They often lead to searches for values of c that are out of the range provided in the table of the function given in Eq. 11.

After estimating α, A, and B, an iterative sequence is initiated. First, cut-off corrections are found for the moments of $F(t)$. These corrections are used to find ϕ_i, and the ϕ_i are used to find α, A, and B. If the new values of α and A agree within an arbitrarily chosen difference, EPS, CONTRL outputs the results and goes on to the next higher MD; otherwise the iteration continues. In practice, EPS is automatically set to 0.0005, and with reasonable values of exponential depression, the program rarely loops more than two times. In other words, the first calculation of the cut-off corrections leads to parameters that agree with the final answer within the value of EPS. Thus, iterative looping serves only to verify the self-consistency of the results. For each successively higher MD, the previously cut-off corrections are used to find the first estimates of α, A, and B, and the iterative sequence is again initiated.

At the CODE position the user may choose to use a new exponential depression, shift the time origin used for calculating the moments of E, thus shifting the data, or choose to enter an entirely new set of decay data. He may also plot the data and the most recent analysis, dump the moments and other internal variables, set a new EPS, or terminate execution.[1]

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[1]Listings of this program will be made available to interested investigators. Requests should be addressed to Dr. Small. The program will be supplied simply as a listing or, if a DEC compatible (RX01) floppy disk is included with the request, the program can be sent on the disk in either RSX-11 or RT-11 format.

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