ANALYSIS OF FLUORESCENCE DECAY CURVES
BY MEANS OF THE LAPLACE TRANSFORMATION

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ABSTRACT   A computational procedure is described for the analysis of fluorescence decay data convolved with a lamp flash of finite width. The computer program calculates the ratio of the Laplace transforms of the decay and the lamp flash for different values of s to give the transforms of the impulse response for each value of s. These are set equal to the analytical Laplace transforms of the decay law involved. Solution of the nonlinear simultaneous equations yields the desired decay parameters. The method can be modified to analyze data that contains a component due to scattered light and can also provide essential information regarding transit time changes of the photomultiplier with changes in emission wavelength. The method was tested by the analysis of real and simulated data. The accuracy of the analysis depends on the degree of correlation among the parameters.

INTRODUCTION

The analysis of fluorescence decay curves is a problem which has attracted much attention in recent years due to the increasing interest in fluorescence decay measurements as a sensitive tool for solving chemical and biophysical problems (1–6).

In the photon counting (7, 8) and sampling (9) techniques the fluorescence is initiated by a relatively short flash of light and the fluorescence intensity is monitored as a function of time yielding the decay curve. The time scale of fluorescence decay is usually in the nanosecond region. Due to the finite width of the lamp flash and to instrumental response time the lamp width obtained in an experiment is usually not negligible compared with the experimental decay curve. The observed fluorescence decay is related to the impulse response (i.e. the fluorescence decay which would have been obtained if the lamp flash was infinitely short) by convolution (Eq. 1):

$$F(t) = \int_0^t E(t - u)I(u)\mathrm{d}u, \tag{1}$$

where $F(t)$ is observed fluorescence decay function, $E(t)$ is observed lamp flash, and $I(t)$ is impulse response function.

In the simplest case the impulse response is a single exponential decay function. In the general case multicomponent decay laws are involved which need not be exponential functions. In many cases of interest, however, the impulse response is given by a sum of exponential terms or may be accurately approximated by such a sum. In

addition to the trivial case of heterogeneity (in which more than one fluorophore is excited) this category includes excited state reactions in the general sense, i.e. any kind of process involving the excited fluorophore and resulting in light emission from a fluorophore which has not been excited directly. Excimer formation, excited state proton transfer, energy transfer and solvent relaxation are some examples (10). If a multiexponential decay law is assumed for the general case, the impulse response is given by:

$$I(t) = \sum_{i=1}^{n} A_i e^{-t/\tau_i}. \tag{2}$$

The A_i's and τ_i's appearing in Eq. 2 are the amplitudes and decay constants, respectively, of the ith component.

The aim of the analysis is to deconvolve the decay function of Eq. 1 and to evaluate the $2n$ parameters of Eq. 2 (nA_i's and $n\tau_i$'s) using the observed decay curve and the experimental lamp flash. Several methods have been developed for the analysis of multiexponential decay data (11–17). The accuracy with which this can be done highly depends on the degree of correlation among the parameters.

Helman (18, 19) has described the use of the Laplace transformation for the analysis of fluorescence decay data. His treatment was restricted to single exponential decay and to the case of excimer formation. The aim of the present paper is to describe a general approach for the analysis of multiexponential decays using the Laplace transformation. The procedure to be described not only provides a simple algorithm for solving for single or double exponentials but also has provision for accounting for a scattering component in the decay curve and for time shifts of the decay relative to the exciting lamp flash. These time shifts are caused by the energy-dependent transit time in the photodetector (20).

THEORY

The Laplace Transform

The Laplace transform ($M(s)$) of a function ($M(t)$) is defined as (21)

$$M(s) = L[M(t)] = \int_0^{\infty} M(t) \cdot e^{-st} dt \, (s \geq 0). \tag{3}$$

The Laplace transform, being an integral, is a linear operator and so:

$$L[aM(t) + bN(t)] = aM(s) + bN(s). \tag{4}$$

The Laplace transform of an exponent can easily be shown to be:

$$L[A \cdot e^{-t/\tau}] = \frac{A}{s + 1/\tau}. \tag{5}$$

A very useful theorem involving the Laplace transformation is the convolution theorem which states:

$$F(s) = L\left[\int_0^t E(t - u) \cdot I(u)du\right] = L[E(t)] \cdot L[I(t)] = E(s) \cdot I(s). \quad (6)$$

Thus the convolution existing between $F(t)$, $E(t)$, and $I(t)$ in the time domain is converted to a simple product in the s domain. By evaluating the Laplace transforms of a decay curve and of the corresponding lamp flash the Laplace transform of the impulse response may be evaluated from the relation:

$$\frac{F(s)}{E(s)} = \frac{E(s) \cdot I(s)}{E(s)} = I(s). \quad (7)$$

In the general case where the impulse response is a sum of n exponentials (see Eq. 2) $I(s)$ has the form:

$$\frac{F(s)}{E(s)} = I(s) = \sum_{i=1}^{n} \frac{A_i}{s + 1/\tau_i}. \quad (8)$$

By evaluating $I(s)$ in this way for $2n$ different values of s, a set of $2n$ equations is obtained, the solution of which yields the desired n amplitudes and n decay constants. In principle any such set of $2n$ different s values can be used to reach this goal, however, as will be discussed, some general considerations regarding the s values may be made to improve the accuracy of the calculated parameters. The solutions of Eq. 8 in the cases of single and double exponential impulse responses are described in the appendix.

Cut-Off Correction

The Laplace transform was defined (see Eq. 3) from zero time (which may be any arbitrary time prior to the lamp flash) to $t = \infty$. The experimental decay curve, however, is never collected to infinite time and does not, in the general case, vanish at the last data channel T (in the instrument used in this laboratory the decay curve is stored in 512 channels of a multichannel analyzer). The lamp flash, on the other hand, usually has a short duration compared to the fluorescence decay. At time T the lamp intensity is, therefore, negligible compared with the decay. The Laplace transform of the impulse response appearing in Eq. 7 thus becomes:

$$I(s) = \frac{F(s)}{E(s)} + \delta I(s), \quad (9)$$

where $\delta I(s)$ is a correction factor given by:

$$\delta I(s) = \frac{1}{E(s)} \int_T^{\infty} F(t) \cdot e^{-st}dt. \quad (10)$$

For an n components decay curve this correction factor will have the form (since the lamp flash ends before time T the decay curve from that time on will be a sum of exponentials):

$$\delta I(s) = \frac{1}{E(s)} \int_T^\infty \sum_{i=1}^n C_i e^{-t/\tau_i} \cdot e^{-st} dt = \frac{1}{E(s)} \sum_{i=1}^n \frac{C_i}{s + 1/\tau_i} e^{-(s+1/\tau_i)T}. \quad (11)$$

C_i, being the contribution of the ith component to the observed decay function at channel T, is given by:

$$C_i = \int_0^T A_i \cdot e^{-t/\tau_i} \cdot E(T - t) dt = A_i \int_0^T E(t) \cdot e^{-(T-t)/\tau_i} dt$$

$$= A_i e^{-T/\tau_i} \int_0^T E(t) \cdot e^{t/\tau_i} dt = A_i e^{-T/\tau_i} \int_0^\infty E(t) \cdot e^{t/\tau_i}$$

$$= A_i e^{-T/\tau_i} \cdot E(-1/\tau_i). \quad (12)$$

$E(-1/\tau_i)$ is the Laplace transform of the lamp flash for the value $-1/\tau_i$ of s.[1]

By introducing these expressions for the C_i's into Eq. 11, the set of equations 8 becomes:

$$\sum_{i=1}^n \frac{A_i}{s + 1/\tau_i} = I(s) = \frac{F(s)}{E(s)} + \sum_{i=1}^n \frac{A_i e^{-(s+2/\tau_i)T}}{s + 1/\tau_i} \cdot \frac{E(-1/\tau_i)}{E(s)}. \quad (13)$$

The second term on the right-hand side is the cut-off correction term, and may be introduced into the equation by an iterative procedure. An approximate set of parameters (A_i's and τ_i's) is calculated from the experimental data using the approximate equations:

$$\frac{F(s)}{E(s)} \sim \sum_{i=1}^n \frac{A_i}{s + 1/\tau_i}. \quad (14)$$

The values of the parameters thus obtained are used to calculate the cut-off corrections which are added to Eq. 13. The new set of equations thus obtained are solved and this process is repeated until a self-consistent set of parameters is obtained.

The Laplace transformation, by nature of its definition tends to reduce the weight of data points at long times as compared with shorter times, thus automatically "ex-

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[1] Strictly speaking the Laplace transformation was defined for $s \geq 0$ but due to the fact that the function $E(t)$ vanishes for all times beyond T the Laplace integral is finite for any finite value of s and the transforms for negative s values can be calculated and used.

ponentially depressing" (14) the data. This largely reduces the number of iterations needed to get convergence of the parameters by the procedure described above.

A simplified procedure for obtaining the cut-off corrections uses values at the long time "tail" of the experimental decay curve to evaluate the C_i's appearing in Eq. 11 rather than calculate them from Eq. 12. To reduce error due to the photon counting noise present in the data these values are averaged over 10 channels in each case (or the long time "tail" of the decay may be smoothed). For a single component analysis the (smoothed) reading at the last data channel is used as the value of C. For two-component analyses the values at two data points, separated by about 50 channels, may be used along with the known (approximate) parameters to evaluate the contribution of each component at the last data channel T, i.e. the C_i's.

This method for cut-off correction assumes that the decay at the long time edge is a sum of exponentials (i.e. that the lamp flash vanishes before that time). This requirement is usually fulfilled except in cases of very short decay times or of lamps with a bad time profile. If this requirement is not fulfilled the accuracy of the cut-off correction might be damaged. The importance of using a lamp with a suitable time profile (i.e. which is very low compared with the fluorescence in the tail) cannot be over emphasized for the analysis of multiexponential data. It was found that for two-component analysis it is usually sufficient that the lamp intensity be 1/100 that of the decay at the long edge of the decay curve.

The use of the simplified cut-off correction method has several advantages over the rigorous cut off correction discussed earlier. The main advantage is that it largely reduces the time of analysis as it does not involve the integrals $E(-1/\tau_i)$ appearing in Eq. 13 so the time for each iteration, as well as the number of iterations is largely reduced (the latter is usually about three to five).

The inaccuracy introduced into the calculated parameters when the C_i's are computed by the "simple" method is, for typical data, at least one order of magnitude smaller than the one caused by the photon counting error inherent in the data and so its influence on the accuracy of the analysis is very small. Moreover, for typical experimental data the accuracy of C_i's calculated by this method is better than that of C_i's calculated by the rigorous convolution method. This is due to the fact that evaluating accurate C_i's by convolution requires very precise knowledge of the parameters (especially the τ_i's) and in order to achieve the same degree of accuracy as in the "simple" method (which is 2–3%) the τ_i's have to be known to within a fraction of a percent. This is rarely the case with noisy experimental data.

Exponential Expansion

The Laplace transform analysis automatically "exponentially depresses" the data thus largely reducing the number of iterations needed for convergence. In some cases, however, the analysis can be improved by applying exponential expansion. This is done by multiplying both the decay and the lamp flash by $e^{t/T}$ (where T, the expansion constant is larger than each of the τ_i's involved in the decay) and Laplace transforming

these new functions:

$$F'(s) = \int_0^{\infty} F(t) \cdot e^{t/T} \cdot e^{-st} = \int_0^{\infty} F(t) \cdot e^{-(s-1/T)t} dt = F(s - 1/T), \quad (15)$$

$$E'(s) = \int_0^{\infty} E(t) \cdot e^{t/T} \cdot e^{-st} = E(s - 1/T). \quad (16)$$

By dividing one gets (by analogy with Eq. 8):

$$\frac{F'(s)}{E'(s)} = \sum_{i=1}^{n} \frac{A_i}{s - 1/T + 1/\tau_i} = \sum_{i=1}^{n} \frac{A_i}{s + 1/T_i}, \quad (17)$$

where T_i is defined by:

$$T_i = T\tau_i/(T - \tau_i). \quad (18)$$

This set of equations 17 is mathematically identical to the set of equations 8, and are solved using the same procedure to yield the n amplitudes and n T_i's. The values of the decay constants, τ_i's, are then calculated from these T_i's using the relation:

$$\tau_i = T_i T/(T + T_i). \quad (19)$$

The advantage of this approach is that the relative separation among the T_i's is larger than that among the τ_i's and the resolution of the analysis for close decay constants is improved. This is shown as follows. The "fractional difference" between two decay constants τ_i and τ_j, which is an indicator for their separation is:

$$(\tau_i - \tau_j)/\tau_j \qquad \tau_i > \tau_j. \quad (20)$$

The analogous expression for the T_i's may be treated by substituting the T_i's according to Eq. 18. One then gets:

$$(T_i - T_j)/T_j = [(\tau_i - \tau_j)/\tau_j] \cdot [T/(T - \tau_i)]. \quad (21)$$

The second term on the right-hand side is larger than 1, as τ_i is always positive, and the fractional separation of the T_i's is better than that of the τ_i's, allowing close decay constants to be better resolved and analyzed by the program.

As this procedure expands the "tail" both of the decay and of the lamp flash the requirement of having a negligibly low lamp tail is more crucial. This method, therefore, yields satisfactory results only when the decay constants involved are quite long compared with the lamp flash.

In many cases of interest the experimental decay curve contains scattered exciting light. This is true for studies of macromolecules, membranes or cells especially when the emission wavelength of the fluorophore is close to the excitation wavelength.

For decay data which contain scattered lamp light the decay function assumes the form:

$$F(t) = \int_0^t E(t - u) \cdot I(u)\,du + C \cdot E(t). \tag{22}$$

C, the scatter constant, defines the amount of scattered lamp light. The Laplace transform of the decay will now be:

$$F(s) = E(s) \cdot I(s) + C \cdot E(s), \tag{23}$$

and the set of equations 8 become

$$\frac{F(s)}{E(s)} = I(s) + C = \sum_{i=1}^{n} \frac{A_i}{s + 1/\tau_i} + C. \tag{24}$$

By calculating the Laplace transforms of the decay and the lamp for $2n + 1$ different values of s, a set of $2n + 1$ equations is obtained. Solution of the equations gives the decay constants, the amplitudes, and the scatter constant. The solutions of Eq. 24 for single and double exponential decays are described in the Appendix. When generating the calculated decay curve the scatter is added so that the experimental and calculated curves can be compared. Strictly speaking the method described above only applies to scattered light having the same wavelength as the fluorescence.

Time Shifts

The transit time of a photomultiplier is usually dependent upon the energy of the photon incident on the photocathode, as well as on other experimental conditions (20) (the position of the photocathode on which the photon is incident being one). This distortion in the time profile of the lamp flash may be well approximated by a relative time shift of the decay curve with respect to the experimental lamp flash. Significant errors in the analysis can occur if this shift is not taken into account.

For a decay curve which has been shifted to longer time the convolution is:

$$F(t + Q) = \int_0^t E(t - u) \cdot I(u)\,du, \tag{25}$$

where Q is the shifting constant. A well-known theorem describes the Laplace trans-

form of a shifted function (21):

$$L[M(t + Q)] = e^{-Qs}L[M(t)] = e^{-Qs} \cdot M(s). \tag{26}$$

By applying this theorem to $F(t)$ in Eq. 25 one gets:

$$F(s) = E(s) \cdot I(s) \cdot e^{-Qs}, \tag{27}$$

and hence:

$$\frac{F(s)}{E(s)} = e^{-Qs} \cdot I(s) = e^{-Qs} \cdot \sum_{i=1}^{n} \frac{A_i}{s + 1/\tau_i}. \tag{28}$$

By evaluating the Laplace transforms of the decay curve and of the lamp flash for $2n + 1$ different values of s and solving the set of equations 28 thus obtained, the amplitudes, decay constants and the shift constant are derived. The solution of Eq. 28 for a shifted single exponential decay is described in the Appendix.

COMPUTER ALGORITHM

The steps involved in the analysis of an n component decay curve are summarized in the following scheme:

(*a*) Laplace transforms of the decay curve and of the lamp flash are calculated for $2n$ different values of s using trapezoidal integration (see the next section for choice of s values). The ratios of these Laplace transforms are calculated to yield $2n$ approximate Laplace transforms of the impulse response (see Eq. 14).

(*b*) The set of $2n$ equations are solved to yield the first approximations for the n decay constants and n amplitudes.

(*c*) These $2n$ parameters are used to evaluate the $2n$ cut off corrections for the Laplace transforms of the impulse response according to Eq. 11. Each of these corrections is added to the corresponding $I_o(s)$ to give a corrected set of $2n$ equations.

(*d*) The corrected equations are solved to yield corrected values for the decay times and amplitudes.

(*e*) The difference between the values of the parameters calculated in the last two loops is determined and steps *c* and *d* are repeated until the difference reaches a prechosen negligible value. Usually no more than four loops are required.

(*f*) When the iteration is complete the program prints out the values for the parameters and convolves the parameters with the experimental lamp flash when instructed to do so. The convolved theoretical curve may then be compared with the experimental decay curve. We use a fast convolution method which has been described in the literature (16).

RESULTS AND DISCUSSION

Data analysis was carried out with a Hewlett-Packard 2100 minicomputer (Hewlett-Packard Co., Palo Alto, Calif.) interfaced with a multichannel pulse height analyzer

(MCA) which has 1024 channels. The MCA is part of a single photon counting fluorescence decay instrument. It has a display unit which provides a convenient way of examining curves generated by the Laplace program and comparing them with experimental decay curves. The computer is interfaced with a digital plotter so that permanent records of experimental or calculated curves may be obtained.

Choice of S Values

It has been found that values for s close to 0, 0.01, 0.02, and 0.03 are usually suitable for analysis of double exponential decay systems. This represents a compromise based on the following considerations. The errors involved in the solution of the simultaneous equations 8 decrease as the numerical divergence in the values chosen for s increases. On the other hand large values of s tend to reduce the significance of τ_i in Eqs. 8 and thus make these equations less sensitive to the decay times. In addition the use of large values of s ($s > 0.1$) in the Laplace transform gives excessive weight to the counts in low numbered channels and small lamp shifts or distortions in the data may over influence the values of the parameters obtained.

The units used for the τ_i's in the calculations are channels (of the MCA). It was found that the best numbers to use for s are those of the same order of magnitude as the expected values for $1/\tau$ (in channels^{-1}). Thus s values are chosen so that $s \sim (C/\tau)$ where C is the instrument's time calibration (nanoseconds/channel) and τ is

TABLE I

EFFECT OF s' ON THE ANALYSIS OF DOUBLE EXPONENTIAL DECAY

Theoretical value	A_1	A_2	τ_1	τ_2
	0.30	0.30	4.00	10.00
Recovered with s'				
0.003	0.31	0.28	4.20	10.17
0.005	0.31	0.29	4.09	10.10
0.007	0.31	0.29	4.05	10.07
0.01	0.30	0.30	4.03	10.05
0.012	0.30	0.30	4.00	10.04
0.014	0.30	0.30	3.99	10.02
0.018	0.30	0.30	3.97	10.00
0.02	0.30	0.30	3.96	10.00
Theoretical value	0.30	0.30	4.00	6.00
Recovered with s'				
0.003	0.59	0	5.10	−23.19
0.005	0.38	0.22	4.22	6.32
0.007	0.36	0.24	4.18	6.25
0.01	0.33	0.27	4.08	6.13
0.012	0.31	0.30	3.99	6.03
0.015	0.30	0.30	3.96	6.01
0.018	0.30	0.30	3.97	6.01
0.02	0.30	0.30	3.97	6.03

the average decay time in nanoseconds. The program has an input of s'. It then selects 0, s', $2s'$, and $3s'$, as the values of s. Thus if s' is chosen as 0.01 the program will use 0, 0.01, 0.02, and 0.03 for the four values of s required to solve a double exponential decay.

The effect of s' on the analysis of a double exponential decay with well separated lifetimes as well as with more closely correlated lifetimes is indicated in Table I. The decay curves with amplitudes of 0.3 and decay times of 4 and 6 ns in one case and 4 and 10 in another were generated in the computer, the decay curve was numerically convolved with a typical experimental lamp flash. For each channel, a random number was selected from a Gaussion distribution of numbers with a mean of zero and a standard deviation of one, and was multiplied by the square root of the counts in that channel. This was then added to the number of counts in that channel to simulate a decay curve with photon counting noise.

As can be seen from Table I the choice of s values was more critical when the parameters were closely correlated than when they were far apart. The optimal s' values depend on the parameters. In the analysis of real data it is convenient to start by using $s' = 0.01$. If the decay constants are correlated (one is less than twice the other) than several other values for s', between 0.003 and 0.02 may be used and the best set of parameters chosen by testing the fit of the calculated curve to the experimental one using the weighted root mean square deviation[2] or the autocorrelation function of the residuals (16). Another approach is to use $s' = 0.01$ to obtain the first set of parameters and then carry out simulations to find the best s' to use for this particular set, thus refining the results obtained. For single exponential decay the choice of s' is not critical.

Accuracy to be Expected

The accuracy to be expected in the values of the parameters obtained by the Laplace analysis, as with other methods of analysis, depends on the degree of correlation among them.

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[2] The weighted root mean in square deviation, WRMS, was defined as:

$$\text{WRMS} = \frac{1}{n-1} \left[\frac{\displaystyle\sum_{i=1}^{n} \left(\frac{F_o(i) - F_c(i)}{F_o(i)} \right)^2 \cdot F_o(i)}{\displaystyle\sum_{i=1}^{n} F_o(i)} \right]^{1/2}$$

$$= \frac{1}{(n-1)\left(\displaystyle\sum_{i=1}^{n} F_o(i)\right)^{1/2}} \cdot \left[\sum_{i=1}^{n} \frac{(F_o(i) - F_c(i))^2}{F_o(i)} \right]^{1/2},$$

where $F_o(i)$, $F_c(i)$ are the observed and calculated fluorescence intensities, respectively, at channel i. n is the number of channels over which the fit is tested (usually covering 300–500 channels).
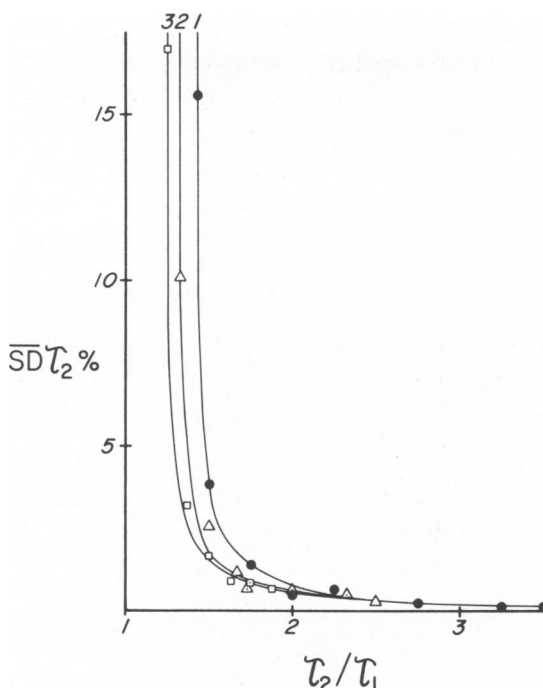
FIGURE 1 The standard deviation obtained for τ_2, the longer of the two decay constants of synthetic double component decay curves, as a function of the ratio of the two decay constants. Curve 1, $\tau_1 = 4$ ns; curve 2, $\tau_1 = 6$ ns; curve 3, $\tau_1 = 8$ ns.

Three sets of experiments were simulated to study this dependence. In each set τ_1 was kept constant while the accuracy of analysis was determined as a function of τ_2. This was done in the following way: A double exponential decay was generated in the computer with about 40,000 counts in the maximum channel, using an experimental lamp flash. Photon counting noise was simulated and added to the decay curve using the procedure described above. The convolved curve was analyzed for the parameters by the Laplace method. This procedure was repeated 20 times for each set of parameters. The standard deviation between the calculated and known decay times was computed in each case and the average standard deviation for the 20 experiments was obtained. τ_2 was then changed and all the steps described above repeated. The results for the simulations of the following sets of parameters are shown in Fig. 1 ($A_1 = A_2 = 0.3$ in all cases): set 1, $\tau_1 = 4$ and $\tau_2 = 5.7$–15 ns. Set 2, $\tau_1 = 6$ and $\tau_2 = 8$–15 ns, and in set 3, $\tau_1 = 8$ and $\tau_2 = 10$–15 ns.

When τ_2 is more than twice τ_1, an accuracy of 1% or better in the standard deviations can be achieved. As τ_2 approaches τ_1 in value a dramatic increase in the standard deviations is apparent. As would be expected the increased error as the two life-times become closer is greater for the shorter lifetimes. These results are in qualitative accord with the theoretical predictions of Isenberg (22).

Simulation studies of this type can give useful insight in regard to the degree of accuracy that can be achieved. Similar experiments have revealed that the accuracy obtained in the analysis depends on the relative amplitudes of the components. Also, better accuracy is obtained when the amplitudes are opposite in sign than when they are of the same sign.

Analysis of Real Data

To evaluate the program with real data, decay curves of 9-cyanoanthracene and β-naphthol were obtained under conditions where both compounds show single exponential decays. The measurements were done by the method of single photon counting (7, 8). Fig. 2 shows the experimental lamp flash and decay curve as well as the result of the Laplace analysis for a single exponential. The residuals[3] between the real and theoretical curve and the autocorrelation function of the residuals (16) indicate a good fit for a single exponential decay. A decay time of 10.3 ns and an amplitude of 0.411 was obtained for 9-cyanoanthracene. A similar fit for a single exponential decay of 4.84 ns and amplitude of 0.467 was obtained with β-naphthol.

The two decay curves were added in equal proportions and the results of a double exponential analysis using various values of s' are summarized in Table II. The best set of parameters obtained in this analysis, as judged by the WRMS (see Table II) and by the autocorrelation function of the residuals was: $\tau_1 = 4.72$, $A_1 = 0.473$, $\tau_2 = 10.38$, $A_2 = 0.411$, in good agreement with the expected values. Fig. 3 shows the experimental and calculated curve as well as the residuals and the autocorrelation function of the residuals for this set of parameters.
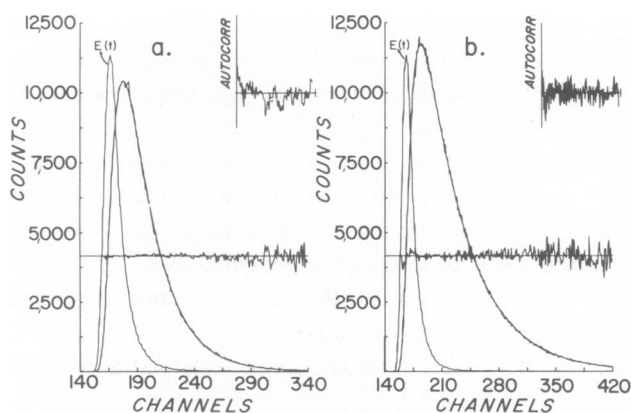

FIGURE 2 Experimental and computed fluorescence decay curves, as well as the lamp flash used for excitation, $E(t)$. The residuals and the autocorrelation of the residuals function show good fits for single exponential decay. Timing calibration 0.204 ns/channel. (a) β-naphthol in aqueous solution, pH = 7. τ = 4.84 ns, A = 0.467. (b) 9-cyanoanthracene in cyclohexane. τ = 10.32 ns, A = 0.411.

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[3] The residuals are given by: $R(i) = [F_o(i) - F_c(i)]/F_o(i)$.

TABLE II
ANALYSIS OF MIXED EXPERIMENTAL DECAY CURVES*

s'	τ_1 ns	A_1	τ_2 ns	A_2	WRMS $\cdot 10^5$
0.007	4.83	0.487	10.49	0.395	4.4194
0.008	4.79	0.481	10.45	0.401	4.4085
0.009	4.72	0.473	10.38	0.411	4.4049
0.010	4.63	0.462	10.30	0.423	4.4165
0.011	4.57	0.454	10.24	0.431	4.4387
0.012	4.49	0.444	10.17	0.442	4.4847

*β-naphthol (τ_1 = 4.84 ns, A_1 = 0.467) and 9-cyanoanthracene (τ_2 = 10.32 ns, A_2 = 0.411) mixed in a 1:1 ratio.

Results obtained by the Laplace analysis of mixtures of the two single exponential decay curves in different proportions are shown in Table III. s' = 0.01 was used for all these analyses. The exponential expansion procedure outlined previously was also used. Comparison of the results obtained with and without the use of this technique shows that in most of the cases studied the exponential expansion improves the results.

Determination of the Shift Correction

The error introduced into the analysis by failure to introduce the shift correction is illustrated in Fig. 4 a which shows the result of an analysis of a 9-cyanoanthracene decay with excitation at 340 nm and emission at 450 nm. The lamp was not shifted prior to analysis. The result indicates a poor fit for a single exponential. Fig. 4 b shows the results of an analysis based on Eq. 28 which include the shift. Three simultaneous equations rather than two are solved and values for Q, τ, and A are obtained (see Appendix). The shift Q was found to be 0.27 ns and a much better analysis for a single exponential is obtained. It has been found that the shift depends on the wavelength and on the area of the photocathode used. Use of the modified Laplace algorithm facilitates the evaluation of the shift correction with the use of a single exponential

TABLE III
ANALYSIS OF EXPERIMENTAL DECAY CURVES
MIXED IN DIFFERENT PROPORTIONS*

Mixing ratio, curve 1/curve 2	Expected amplitudes		Found							
			No exponential expansion				With exponential expansion			
	A_1	A_2	τ_1	A_1	τ_2	A_2	τ_1	A_1	τ_2	A_2
1	0.467	0.411	4.63	0.462	10.30	0.423	4.74	0.472	10.37	0.409
2	0.467	0.205	4.52	0.442	10.00	0.238	4.69	0.466	10.32	0.212
5	0.467	0.082	4.49	0.437	9.51	0.120	4.71	0.466	10.34	0.088
0.5	0.234	0.411	4.61	0.231	10.30	0.417	4.84	0.246	10.43	0.400
0.2	0.093	0.411	4.80	0.101	10.38	0.405	5.19	0.114	10.48	0.391

*β-naphthol (τ_1 = 4.84 ns, A_1 = 0.467) and 9-cyanoanthracene (τ_2 = 10.32 ns, A_2 = 0.411).
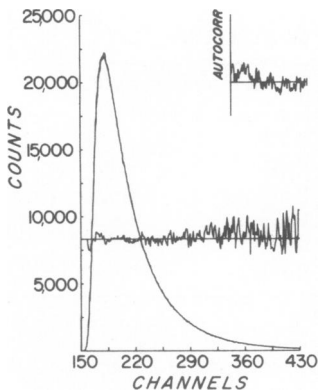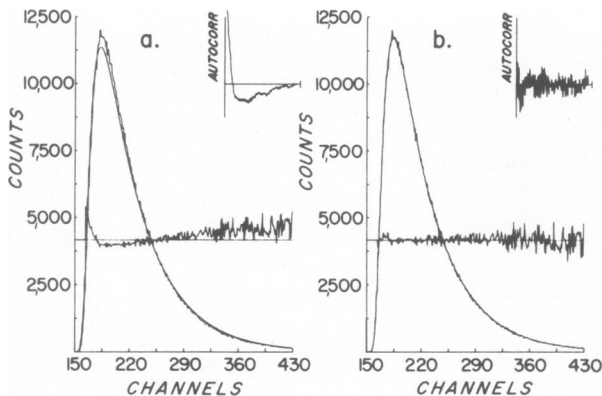
FIGURE 3   FIGURE 4

FIGURE 3   Mixed experimental fluorescence decay data, observed and computed. The data are those for β-naphthol and 9-cyanoanthracene shown in Fig. 2 mixed in 1:1 proportions. An s' value of 0.009 was used in this analysis. An identical lamp flash was used for both experiments.

FIGURE 4   Fluorescence decay curves (experimental and computed) of 9-cyanoanthracene in cyclohexane. Excitation wavelength 340 nm, emission observed at 450 nm. Timing calibration 0.204 ns/channel. (a) Data analyzed using Eq. 8 (time shift not taken into account). Parameters obtained: $\tau = 10.74$, $A = 0.395$. (b) Data analyzed using Eq. 28 (time shift incorporated as a parameter) yielding: $\tau = 10.32$ ns, $A = 0.411$, $Q = 0.27$ ns.
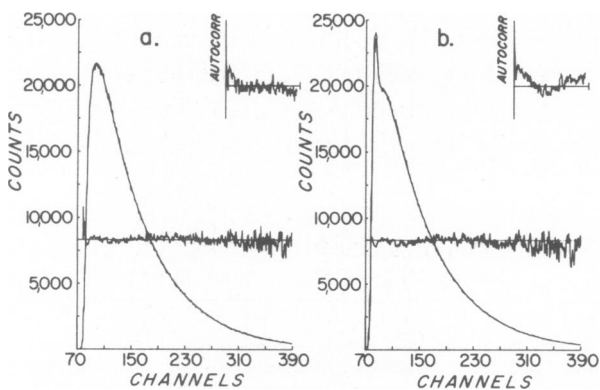

FIGURE 5   Experimental and computed fluorescence decay curves of acridine ($2 \cdot 10^{-5}$ M) in aqueous 0.2 M ammonium nitrate solution, pH = 8.3. Excitation wavelength 355 nm, emission observed at 560 nm. Timing calibration 0.40 ns/channel. (a) Decay curve analyzed for two components. Parameters obtained: $\tau_1 = 3.69$ ns, $A_1 = -0.126$, $\tau_2 = 28.96$ ns, $A_2 = 0.386$. (b) The decay curve to which 0.700 of the exciting lamp flash was added and analyzed using the set of equations 24. The parameters obtained: $\tau_1 = 3.58$ ns, $A_1 = -0.135$, $\tau_2 = 28.90$ ns, $A_2 = 0.387$, $C = 0.703$.

standard. Failure to take the shift into account can lead to serious errors in the estimation of exponential parameters, especially for multiexponential decay curves.

Estimation of Parameters from Decay Curves which Contain a Component Due to Scattered Light

Under some experimental conditions decay curves contain a significant scatter component. To study the accuracy of the analysis in this important case an experimental decay curve was obtained for acridine in aqueous 0.2 N ammonium nitrate solution. Under these conditions a partial excited state proton transfer reaction takes place, as a result of which the observed decay is a double exponential, the two amplitudes being of opposite signs. The decay curve was analyzed and the results are shown in Fig. 5 A. A 70% component of the experimental lamp flash was then added to the decay. Fig. 5 B shows an analysis carried out by the Laplace program modified according to Eqs. 22–24. The parameters are in good agreement with those obtained without scatter and the scattering component was found to be in good agreement with the amount of scatter added to the data.

CONCLUSION

Fluorescence decay measurements are capable of providing detailed kinetic information about excited-state reactions. Good procedures for data analysis are as essential as the proper instrumentation. Some of the problems involved have been discussed by Knight and Selinger (23). The detailed papers of Isenberg (12, 14, 22) have clearly outlined the problems involved and described the method of moments as an excellent computer approach to the problem. The method of nonlinear least squares can also be used for analysis as outlined in detail by Grinvald and Steinberg (16).

The Laplace method described here is straight forward in concept and could be adapted to a variety of decay laws. It is simple to program and requires little computer core or time, while yielding excellent results. It should be of value to have a variety of techniques available for the analysis of fluorescence decay data.

APPENDIX

$2n$ equations of the type shown by Eq. 8 are needed to analyze an n component decay curve. As was mentioned in the text one value, s', is asked for by the program and the set $0, s', 2s', \ldots (2n - 1)s'$ is then used to obtain and solve Eq. 8.

(a) For a single exponential decay the equations assume the form:

$$I(0) = A/(1/\tau),$$
$$I(s') = A/(s' + 1/\tau).$$

The solution of these equations is:

$$\tau = (I(0) - I(s'))/s' \cdot I(s'),$$

$$A = I(0)/\tau.$$

(b) For a single exponential decay with time shift Eqs. 28 become:

$$I(0) = A/(1/\tau),$$
$$I(s') = A/(s' + 1/\tau)\cdot e^{-Qs'},$$
$$I(2s') = A/(2s' + 1/\tau)\cdot e^{-2Qs'},$$

and the solutions of these equations are:

$$\tau = \cfrac{1}{s'\left[\left(\cfrac{I(0)\cdot I(2s')}{I(0)\cdot I(2s') - I^2(s')}\right)^{1/2} - 1\right]},$$

$$A = I(0)/\tau,$$

$$Q = \frac{1}{s'}\cdot \ln\left[\frac{I(0)}{I(s')}\cdot\frac{1/\tau}{s' + 1/\tau}\right].$$

(c) For a single component with light scatter the equations are:

$$I(0) = [A/1/\tau)] + C,$$
$$I(s') = [A/(s' + 1/\tau)] + C,$$
$$I(2s') = [A/(2s' + 1/\tau)] + C,$$

and the solutions:

$$\tau = \frac{1}{2s'}\left(\frac{I(s') - I(0)}{I(2s') - I(s')} - 1\right)$$

$$A = \frac{[I(0) - I(2s')]}{2s'}\cdot\left[\frac{1}{\tau}\cdot\left(\frac{1}{\tau} + 2s'\right)\right]$$

$$C = I(2s') - A/(2s' + 1/\tau).$$

(d) For two components the four equations are:

$$I(0) = (A_1/1/\tau_1) + (A_2/1/\tau_2),$$
$$I(s') = [A_1/(s' + 1/\tau_1)] + [A_2/(s' + 1/\tau_2)],$$
$$I(2s') = [A_1/(2s' + 1/\tau_1)] + [A_2/(2s' + 1/\tau_2)],$$
$$I(3s') = [A_1/(3s' + 1/\tau_1)] + [A_2/(3s' + 1/\tau_2)].$$

For presenting the solutions for these equations one may define:

$$V_1 = 2s'^2[I(s') - 2I(2s')],$$

$$V_2 = 2s'[I(s') - I(2s')],$$

$$V_3 = I(2s') + I(0) - 2I(s'),$$

$$V_4 = 3s'^2[I(s') - 3I(3s')],$$

$$V_5 = 3s'[I(s') - I(3s')],$$

$$V_6 = 3I(s') - 2I(0) - I(3s'),$$

$$V_7 = (V_3 \cdot V_4 + V_1 \cdot V_6)/(V_3 \cdot V_5 + V_2 \cdot V_6),$$

$$V_8 = (V_1 + V_2 \cdot V_7)/V_3,$$

$$\tau_1 = 2/[V_7 + (V_7^2 - 4V_8)^{1/2}],$$

$$\tau_2 = 2/[V_7 - (V_7^2 - 4V_8)^{1/2}],$$

$$A_1 = \left[\tau_2 \cdot I(s') - \left(\frac{1}{s' + 1/\tau_2}\right) \cdot I(0)\right] \Big/ \left[\left(\frac{\tau_2}{s' + 1/\tau_1}\right) - \left(\frac{\tau_1}{s' + 1/\tau_2}\right)\right]$$

$$A_2 = (I(0) - \tau_1 \cdot A_1)/\tau_2.$$

(e) In the case of a double exponential decay with scattered exciting light the equations are:

$$I(0) = [A_1/(1/\tau_1)] + [A_2/(1/\tau_2)] + C$$

$$I(s') = [A_1/(s' + 1/\tau_1)] + [A_2/(s' + 1/\tau_2)] + C,$$

$$I(2s') = [A_1/(2s' + 1/\tau_1)] + [A_2/(2s' + 1/\tau_2)] + C,$$

$$I(3s') = [A_1/(3s' + 1/\tau_1)] + [A_2/(3s' + 1/\tau_2)] + C,$$

$$I(4s') = [A_1/(4s' + 1/\tau_1)] + [A_2/(4s' + 1/\tau_2)] + C,$$

By defining:

$$U_1 = 2[I(0) + 3I(2s') - 3I(s') - I(3s')],$$

$$U_2 = 6s'[I(s') + I(3s') - 2I(2s')],$$

$$U_3 = 6s'^2[I(s') + 3I(3s') - 4I(2s')],$$

$$U_4 = 3I(0) + 6I(2s') - 8I(s') - I(4s'),$$

$$U_5 = 4s'[2I(s') - 3I(2s') + I(4s')],$$

$$U_6 = 4s'^2[2I(s') + 4I(4s') - 6I(2s')],$$

$$U_7 = (U_1 \cdot U_6 - U_3 \cdot U_4)/(U_2 \cdot U_4 - U_1 \cdot U_5),$$

$$U_8 = (U_2 \cdot U_7 + U_3)/U_1,$$

one gets as the solutions of the above equations:

$$\tau_1 = 2/[U_7 + (U_7^2 - 4U_8)^{1/2}],$$

$$\tau_2 = 2/[U_7 - (U_7^2 - 4U_8)^{1/2}],$$

$$A_1 = \{[I(0) - I(s')] \cdot K_4 - [I(0) - I(2s')]K_2\}/K_1 \cdot K_4 - K_3 \cdot K_2,$$

$$A_2 = [I(0) - I(s') - K_1 \cdot A_1]/K_2$$

where:

$$K_1 = s' \cdot \tau_1/(s' + 1/\tau_1), \qquad K_2 = s' \cdot \tau_2/(s' + 1/\tau_2),$$

$$K_3 = 2s' \cdot \tau_1/(2s' + 1/\tau_1), \qquad K_4 = 2s' \cdot \tau_2/(2s' + 1/\tau_2).$$

And for the scatter constant one may use the equation

$$C = I(0) - A_1 \cdot \tau_1 - A_2 \cdot \tau_2.$$

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