STUDIES ON THE ANALYSIS OF FLUORESCENCE DECAY DATA BY THE METHOD OF MOMENTS

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ABSTRACT The method of moments, as presented by Isenberg and Dyson (1969; *Biophys. J.* 9:1337) has been shown to be a reliable way of obtaining the amplitudes and time constants of several simultaneously emitting species, even in the presence of an overlapping excitation. Recent improvements in the method include (*a*) a component incrementation test for determining the number of relaxations, (*b*) a procedure, which we call exponential depression, for dramatically improving convergence, and (*c*) a new algorithm for implementing the method of moments on a digital computer with a high degree of flexibility and efficiency. These improvements, as well as new general theory, are described and tested using both synthetic and real experimental data. Component incrementation consists of examining models with increasing numbers of exponential terms. Given adequate precision, we find that an analysis for $N + 1$ components, of data that are actually represented by N components, provides the correct amplitudes and time constants plus an $N + 1$ term with an insignificant amplitude. Exponential depression is a transformation in which the original excitation and fluorescence, $E(t)$ and $F(t)$, are multiplied by $\exp(-\lambda t)$, where λ is an arbitrary parameter. While the convolution is invariant to this transformation, the proper choice of λ greatly reduces the number of iterations necessary to obtain the amplitudes and time constants and may even improve their accuracy. In addition, an appendix by John P. Mullooly presents a statistical analysis of the effect of counting error on the method of moments estimates of fluorescence decay parameters, applicable when data are obtained by the monophoton technique. Formulas are derived that give the approximate precision of the decay parameters for the general case of N exponential components, with calculational details for one and two component systems.

INTRODUCTION

There is currently considerable interest in the measurement of fluorescence decay of proteins, protein conjugates, and nucleic acid conjugates. The decay of a polarized component of emission, for example, is a function of the rotatory diffusion constants of the macromolecule (1–10). Consequently, the analysis of such decay data can yield important physical parameters.

The measurement of a single decay constant for one species, and the analysis of the resultant data, involves little difficulty. However, in many problems, including the study of rotatory diffusion by polarization of fluorescence, one must deal with multicomponent or multiexponential data (6, 11, 12).

The analysis of multiexponential data has intrinsic difficulties, which may be illustrated by the plots shown in Fig. 1. These plots show that two quite different multiexponential curves may look, to the eye, as one curve to a high degree of accuracy and over a relatively large range. The deviation between the two plots where they appear the same is less than the width of the pen line used to represent them. If one of the two curves of Fig. 1 represented an actual set of data, and an attempt were made to determine the parameters of the exponentials by curve fitting, one could make serious errors by using criteria of fit that scientists are accustomed to accept in many other areas of work.

The difficulty of analyzing multiexponential data has often been noted (13, 14). This difficulty is coupled with its widespread importance, since, aside from fluorescence decay, such data appear in a variety of fields, including sedimentation equilibrium in the ultracentrifuge (15), compartmental analysis in physiology (16), kinetics of isotope exchange (17, 18), and the analysis of reaction rates (19).

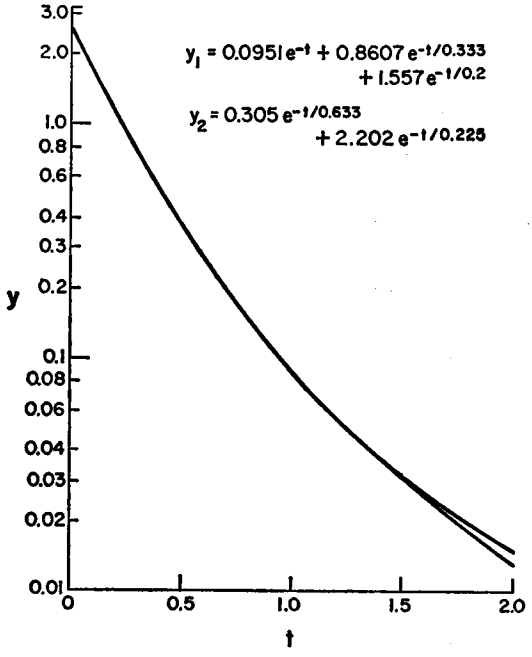


FIGURE 1 Semilogarithmic plots of two quite different exponential functions. For about two decades the plots deviate less than the width of the line used to represent them. Data from reference 13.

It is not surprising, therefore, that numerous attempts have been made to develop adequate means for the analysis of multiexponential data. Many of these attempts have been interesting but, almost without exception, have not found usefulness in the analysis of real data.

In 1969 two of the authors of this paper published a method of moments for the analysis of multicomponent fluorescence decay data (20). This work had a number of precursors, stemming from the paper of Bay (21). Bay's work was aimed at the analysis of radioactive decay experiments, and he noted that, by using the moments of decay data, one could obtain mean decay times without a knowledge of, or previous assumptions about, the delays inherent in the experimental equipment or the resolving time of such equipment. Brody (22) applied this method to the analysis of one-component fluorescence decay data, and Wahl and Lami (23) combined it with a substraction procedure to analyze two-component data. The paper by Isenberg and Dyson (20) presented a more general scheme, one that permits the resolution of multiexponential data (24), and also data whose lifetimes are appreciably shorter than the width of the exciting lamp (10). In that paper, among other things, the importance of the so-called cutoff correction was established for a practical application of the procedure. In a later paper (25), we treated the simpler cases, such as sedimentation equilibrium, where the data may be represented by a linear sum of exponentials without an exciting pulse. In such instances, we have shown that a useful smoothing functional, the mean displaced ratio, may permit an adequate analysis of relatively noisy data.

In the present paper, we shall restrict our discussion to problems of analyzing fluorescence decay data complicated by an overlapping excitation. We shall present new developments in the formalism and programming of the method of moments which have significant practical value in analyzing data. Although much of the discussion and all of the examples are of monophoton fluorometry, the procedures are, at least in principle, applicable to any pulsed fluorometer that provides computer-compatible representations of both the excitation and fluorescence.

STATEMENT OF THE PROBLEM

The problem facing an investigator is to obtain the important physical parameters in f, the response to a delta function excitation. If, for example, f is a sum of exponentials, one wants to know the amplitude α_i and decay constant τ_i for each exponential. The problem is *not* how to fit a given set of data by a sum of exponentials. As has been seen, and as will be further shown, a set of data may be fitted by a sum of exponentials having parameters that are grossly in error, even though the fit may look excellent by standards that workers are accustomed to accept as good in other areas. This may occur when the precision of the data is below the level needed for an adequate analysis. An auxiliary and important subproblem, therefore, is to be able to judge when the data are good enough to be analyzed and to set up criteria for so judging.

THEORY

The observed fluorescence $F(t)$ is the convolution of the exciting lamp flash $E(t)$ and the response to a delta function excitation $f(t)$:

$$F(t) = \int_0^t E(t-u)f(u)\,du = \int_0^t E(u)f(t-u)\,du. \tag{1}$$

We shall assume that $f(u)$ has the form

$$f(u) = \sum_{i=1}^{N} \alpha_i \exp(-u/\tau_i), \tag{2}$$

where α_i and τ_i are the amplitudes and time constants, respectively, of the ith component.

This multiexponential form does not cover all cases of fluorescence decay, but it nevertheless subtends a wide enough set of cases so as to make an analysis important (20).

We define moments by

$$\mu_k = \int_0^\infty t^k F(t)\,dt, \tag{3a}$$

and

$$m_k = \int_0^\infty t^k E(t)\,dt, \tag{3b}$$

and a set of quantities G_s by

$$G_s = \sum_{i=1}^{N} \alpha_i \tau_i^s. \tag{4}$$

It can then be shown that (see reference 20)

$$\begin{aligned}
\mu_0 &= G_1 m_0, \\
\mu_1 &= G_1 m_1 + G_2 m_0, \\
\frac{\mu_2}{2!} &= G_1 \frac{m_2}{2!} + G_2 m_1 + G_3 m_0, \\
&\vdots \\
\frac{\mu_k}{k!} &= \sum_{s=1}^{k+1} Gs\, \frac{m_{k+1-s}}{(k+1-s)!}.
\end{aligned} \tag{5}$$

These are a set of equations that are linear in the parameters G_s. Given the moments of the excitation $E(t)$ and the moments of the fluorescence $F(t)$, one can obtain $G_1, G_2, \cdots$ from Eqs. 5. Knowing $G_1, G_2, \cdots G_{2N}$, one can obtain the decay

times, $\tau_1, \tau_2, \cdots \tau_N$ as the roots of the polynomial equation (see Appendix I)

$$\begin{vmatrix} 1 & \tau & \tau^2 & \cdots & \tau^N \\ G_1 & G_2 & G_3 & \cdots & G_{N+1} \\ G_2 & G_3 & G_4 & \cdots & G_{N+2} \\ \vdots & & & & \\ G_N & G_{N+1} & G_{N+2} & \cdots & G_{2N} \end{vmatrix} = 0. \tag{6}$$

Knowing $\tau_1, \tau_2, \cdots$ one obtains the amplitudes $\alpha_1, \alpha_2, \cdots$ from the set

$$\begin{aligned} G_1 &= \sum_{i=1}^{N} \alpha_i \tau_i, \\ G_2 &= \sum_{i=1}^{N} \alpha_i \tau_i^2, \\ &\vdots \\ G_N &= \sum_{i=1}^{N} \alpha_i \tau_i^N, \end{aligned} \tag{7}$$

obtained from Eq. 4. This procedure formally solves the problem of obtaining the amplitudes and time constants of decay from the experimental data.

Eq. 6 has both theoretical and practical value. Let us first consider a general implication.

Suppose we imagine certain broad categories of methods, of any type, for obtaining the amplitudes and the time constants of fluorescence decay. Such methods may be grouped into two classes. One class formally recognizes that the decay times may be ordered according to their magnitudes. An example is the peeling-off procedure, in which one first attempts to obtain the longest decay time by considering data at very long times when, it is presumed, all decays but one have become negligibly small. Then, by subtraction, one obtains data from which one attempts to determine the next greatest time constant, and so on.

The other category of procedures contains systems that do not distinguish or rank the decays in any formal way. It may be seen that, of such methods, the method of moments, via Eq. 6, offers a particularly simple manner of solving for the decay times, since one could hardly expect to find a simpler formal solution than one yielding N decay times as the roots of a polynomial equation of the Nth degree.

As we show in Appendix I, Eq. 6 may also be used in the well-known Prony procedure for obtaining the parameters of a sum of exponentials. Eq. 6 is, however, more compact than the usual Prony treatment.

RELIABILITY OF THE ANALYSIS

The method of moments will always curve fit a set of data, even when the precision of the data is not good enough to obtain the parameters with accuracy. Figs 1–3

and Table I illustrate this. It may be seen that, by customary standards used in other areas of work, all of the plots appear to be good fits. However, the parameters obtained at the lower levels of precision are not good; for an accurate analysis one must go to the higher range of precision.

These examples demonstrate the distinction emphasized earlier: the substantive problem facing the worker is one of obtaining the parameters of decay; it is not the problem of fitting a curve to a set of data. While the correct parameters always give a good fit, so may incorrect parameters. The blurring of the distinction between the two formulations can lead to the acceptance of inaccurate analyses.

This consideration makes it necessary to establish criteria for having confidence in the decay parameters that are obtained. We believe it to be important to consider such criteria explicitly. Such criteria are of two types, experimental and statistical. Experimentally, aside from ordinary good laboratory practice involving duplicability, we have found the following useful: (*a*) in the low precision range, we find that a variation in precision alters the decay parameters that are obtained; in the high precision range it does not. Therefore a variation in the precision of the data will help one to judge which range one is in. In the monophoton method, for example, this ordinarily means doing experiments at various numbers of total counts. (*b*) One must judge the number of components present. We have found it useful to analyze for a successively higher number of components until one finds that, upon doing so, no significant change occurs at a particular increment. For example, suppose one analyzes for two components and finds that one of them is close to the value obtained in a one-component analysis, while the other has a very low amplitude compared with the first. This would imply that, within experimental error, one component satisfies the data. On the other hand, if one found two components having amplitudes of the same order of magnitude, one would analyze for three components, etc. We shall call this technique component incrementation.

A statistical analysis of reliability is presented by Mullooly in Appendix III. Mullooly obtains expressions for the estimated standard deviation (σ) of the time constants and the amplitudes in terms of the moments of the data. In general, we find that when σ is large, an analysis is unreliable; when the standard deviation is small, the analysis is accurate. Thus, the statistical analysis is important in judging reliability. However, we feel that both statistical and experimental criteria are needed. Real experiments may, and generally will, have noise components in addition to the counting errors assumed in Mullooly's study. Furthermore, the calculation of σ requires higher moments than are needed in an analysis for the decay parameters. To compute such moments accurately one needs a better cutoff correction (see below) than is needed for an analysis. Consequently, the statistical criteria may either overestimate or underestimate the precision of the data. Nevertheless, the statistical analysis provides a significant advance in judging experimental reliability.

CUTOFF CORRECTION

The cutoff error is the error that arises when, in place of Eq. 3*a*, the μ_k are approximated by

$$\mu_k^T = \int_0^T t^k F(t)\, dt, \tag{8}$$

where T is the upper limit of measured times.

Eqs. 5 are not valid for moments defined over a finite span, and we have noted (20) that large errors will be made if the moments are approximated by Eq. 8, even though T is a value that, at first glance, appears to be reasonably large. It is necessary, therefore, to correct the error made because real data extend over only a finite range.

An iterative procedure is used to compute the cutoff correction. One first solves the set of Eqs. 5 using the moments defined by Eq. 8. The $\alpha_1, \alpha_2, \cdots \alpha_N, \tau_1, \tau_2, \cdots \tau_N$ thus obtained are used to find the moment correction from T to infinity as follows: from Eqs. 1–3*a*, noting that $E = 0$ at $t > T_E$ (where $T_E \leq T$) we write

$$\begin{aligned}\delta\mu_k &= \int_T^\infty t^k F(t)\, dt, \\ &= \sum_{i=1}^N \beta_i \int_T^\infty t^k \exp(-t/\tau_i)\, dt = \sum_{i=1}^N \beta_i I_{i,k},\end{aligned} \tag{9}$$

with

$$\beta_i = \alpha_i \int_0^{T_E} E(u) \exp(u/\tau_i)\, du. \tag{10}$$

The values of β_i are obtained by numerical integration, while the integrals $I_{i,k}$ are readily evaluated by recursion:

$$\begin{aligned}I_{i,0} &= \tau_i \exp(-T/\tau_i), \\ I_{i,1} &= T\tau_i \exp(-T/\tau_i) + \tau_i I_{i,0}, \\ &\vdots \\ I_{i,k} &= T^k \tau_i \exp(-T/\tau_i) + k\tau_i I_{i,k-1}.\end{aligned} \tag{11}$$

The new estimates of the moments

$$\mu_k = \mu_k^T + \delta\mu_k$$

are then used to find another set of α_i and τ_i, and from them the next cutoff correction. This sequence is repeated until no change occurs upon additional looping. In

other words, the parameters are improved at each recalculation until a self-consistent set is obtained.

We have found such a procedure to be effective. But for some analyses the number of loops needed becomes large, and the analysis becomes extremely costly in computer time—so much so, in fact, that economic considerations begin to severely limit the practical usefulness of the procedure. In this paper we present a new method of handling the cutoff correction problem, which sharply decreases the number of iterative loops needed in an analysis. We call the procedure exponential depression.

EXPONENTIAL DEPRESSION

For some positive, but arbitrary, parameter λ, multiply Eq. 1 by exp $(-\lambda t)$. Then

$$e^{-\lambda t}F(t) = \int_0^T e^{-\lambda t}E(t-u)f(u)\,du,$$

$$= \int_0^T e^{-\lambda(t-u)}E(t-u)e^{-\lambda u}f(u)\,du, \tag{12}$$

Define

$$F_\lambda(t) = e^{-\lambda t}F(t), \tag{13a}$$

$$E_\lambda(t) = e^{-\lambda t}E(t), \tag{13b}$$

$$f_\lambda(t) = e^{-\lambda}f(t). \tag{13c}$$

Then

$$F_\lambda(t) = \int_0^t E_\lambda(t-u)f_\lambda(u)\,du. \tag{14}$$

In other words, the convolution is invariant to the transformation defined by Eqs. 13. One may, for an arbitrary λ, solve for the parameters in $f_\lambda(t)$ and hence obtain f. By choosing λ properly, $F_\lambda(t)$ becomes much smaller than $F(t)$ for $t \geq T$, so that the cutoff errors in the moments of F_λ are much lower than those of F. The number of loops needed to obtain the cutoff correction is thus sharply reduced, as will be seen in the examples below.

We find that exponential depression has an additional benefit. It depresses the data at long times more than it does at short times. Since the data for large t are relatively noisier than the data at short t, exponential depression can increase the accuracy of an analysis. We note, however, that for sufficiently large λ the accuracy declines with an increase in λ.

EXAMPLES OF ANALYSES

In the following sections we present a number of examples, both as tests and as illustrations of the various points raised in this paper. The examples include both

synthetic and real data. Each has its own advantages. Synthetic data avoid miscellaneous sources of error, such as lamp drift, which may tend to bscure what would otherwise be clear considerations. On the other hand, examples using real data show that the difficulties associated with actual experiments are not great enough to nullify practical applications of the theory.

MATERIALS AND PROCEDURE

Synthetic data were generated using real lamp data for $E(t)$, selecting appropriate parameters α_i, τ_i, calculating $F(t)$, and then superimposing synthetic noise on $F(t)$. The noise is obtained from the random number generator described by Moshman (26). The noise is distributed in a gaussian fashion with a standard deviation equal to the square root of the number of counts in the channel to which the noise is added.

For real data, a monophoton fluorometer, described in the paper by Schuyler and Isenberg (24), was used.

Three substances were used for test samples: quinine bisulfate, carbazole, and anthracene. Runs were also made on samples containing mixtures of carbazole and anthracene.

Quinine bisulfate, purchased from the S. B. Penick Chemical and Co. (Division of CPC International Inc., New York), was recrystallized from water. For quinine, the excitation was selected by a Corning 7-60 filter (Corning Glass Works, Science Products Div., Corning, N. Y.) and the emission by a Baird-Atomic interference filter (Baird-Atomic Inc., Cambridge, Mass.) with a peak at 460 nm and a full width at half-height (FWHH) of 1.1 nm. Quinine was used at 10^{-4} M in 0.1 N H_2SO_4.

Carbazole (Matheson, Coleman and Bell, Cincinnati, Ohio) and anthracene (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) were used without further purification. Carbazole was used at 2.7×10^{-4} M in 95% ethanol and anthracene was 4.7×10^{-5} M in 95% ethanol. Mixed samples of carbazole and anthracene were obtained by mixing 3 parts of the carbazole solution to 1 part of the anthracene solution. Excitation was selected by a Baird-Atomic interference filter with a peak at 325 nm and an FWHH of 2.5 nm, and the emission by a Baird-Atomic interference filter with a peak at 400 nm and an FWHH of 2.0 nm.

STATISTICAL ANALYSES OF PRECISION

Table I shows a variety of cases, both synthetic and real, at varying numbers of total counts. The indicated errors are those calculated by the use of the statistical error equations derived by Mullooly in Appendix III. The features are clear: when Mullooly's equations predict a large error, our analysis usually shows a large deviation from the parameters known to be present. When small errors are predicted, small deviations are found.

The method of moments always curve fits the data, even when the precision of the data is too low to yield a good analysis. Figs. 2 and 3 show plots of synthetic and real two-component data from Table I. Note that in the lower range of precision the fit appears good visually, even though the analysis is bad.

Fig. 4 demonstrates the power of the method of moments to resolve data in the presence of very large amounts of noise. With only 194 counts distributed over 500 channels, the method nevertheless produces a satisfactory time constant for the one component present.

TABLE I

PRECISION VERSUS NUMBER OF COUNTS*

Synthetic data components	Preset parameters		Total counts	Analyzed parameters			
	α	τ		α	$\sigma(\alpha)$	τ	$\sigma(\tau)$
		ns				*ns*	*ns*
1	0.015	19.4	258.650	0.0150	0.00003	19.37	0.04
1	0.0015	19.4	26.786	0.00149	0.000009	19.42	0.12
1	0.00015	19.4	2,831	0.000150	0.000003	20.36	0.38
1	0.000015	19.4	286	0.000018	0.000001	16.95	1.02
2	1.00	9.8	16,732,352	0.995	0.0076	9.82	0.02
Fig. 2 A	2.00	4.0		2.004	0.0056	4.01	0.02
2	0.200	9.8	3,345,533	0.195	0.0033	9.87	0.04
	0.400	4.0		0.405	0.0025	4.04	0.04
2	0.070	9.8	983,152	0.066	0.0020	9.98	0.08
	0.090	4.0		0.093	0.0016	4.21	0.10
2	0.020	9.8	335,100	0.018	0.00100	10.09	0.15
Fig. 2 B	0.040	4.0		0.042	0.00074	4.14	0.11
2	0.003	9.8	46,317	0.0046	0.00043	8.47	0.24
	0.005	4.0		0.0038	0.00019	2.63	0.65
Real	Experimental conditions						
1	Quinine bisulfate		178,677	0.0098	0.00002	19.37	0.05
1	1×10^{-4} M in 0.1 M $(NH_4)_2SO_4$		17,938	0.00099	0.000007	19.42	0.15
1			1,765	0.000096	0.000002	19.76	0.45
1			194‡	0.000011	0.0000007	19.37	1.31
2	Carbazole 2.7×10^{-4}M		2,564,052	0.030	0.00075	9.87	0.06
Fig. 3 A	Anthracene 4.7×10^{-5} M			0.071	0.00062	4.48	0.04
2	Both in 95% ETOH		904,368	0.011	0.00063	10.60	0.14
Fig. 3 B	$\tau_C = 9.6$ ns, $\sigma(\tau_C) = 0.1$ ns			0.038	0.00056	4.30	0.06
2	$\tau_A = 4.6$ ns, $\sigma(\tau_A) = 0.1$ ns		289,266	0.002	0.00016	13.60	0.24
				0.014	0.00014	6.10	0.06
2			52,208	0.0005	0.00011	11.47	0.56
				0.0023	0.00009	5.41	0.21

* $\sigma(\alpha)$ and $\sigma(\tau)$ are the estimated standard deviations of the amplitude and time constant, respectively, computed by the equations of Mullooly in Appendix III.

‡ See Fig. 4.

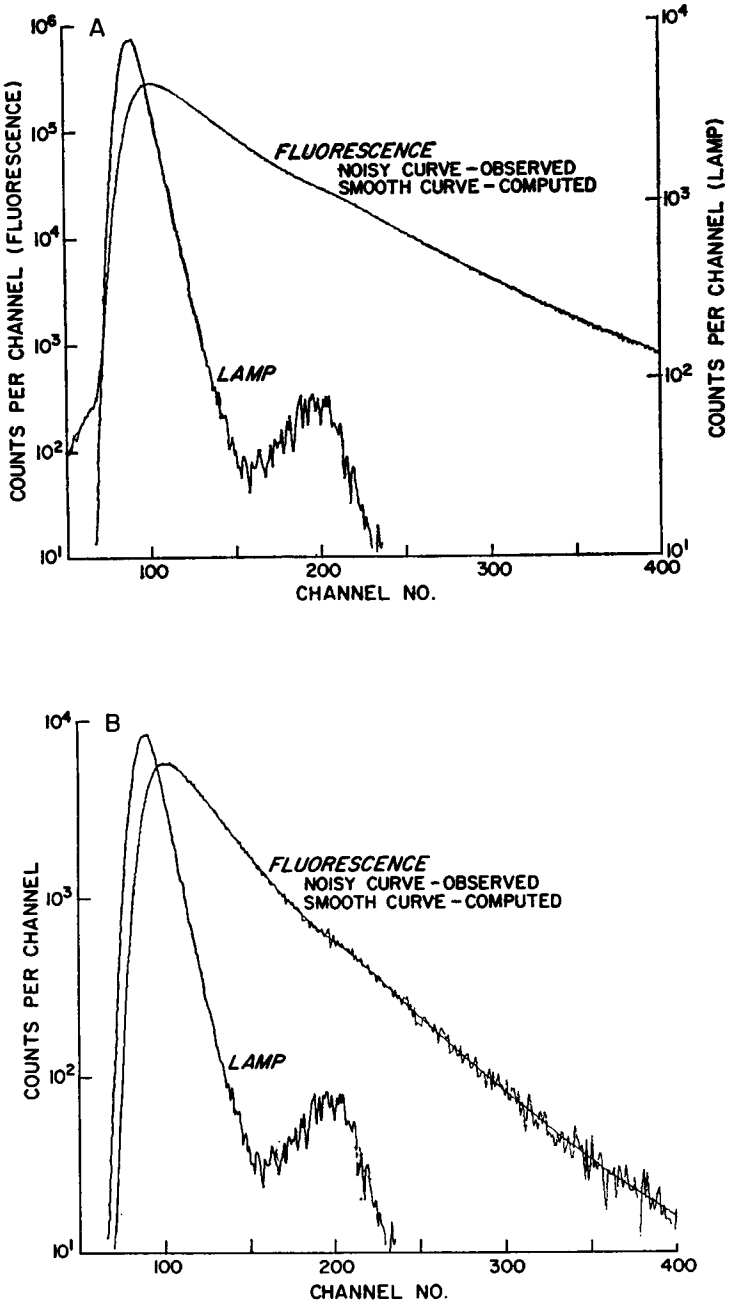
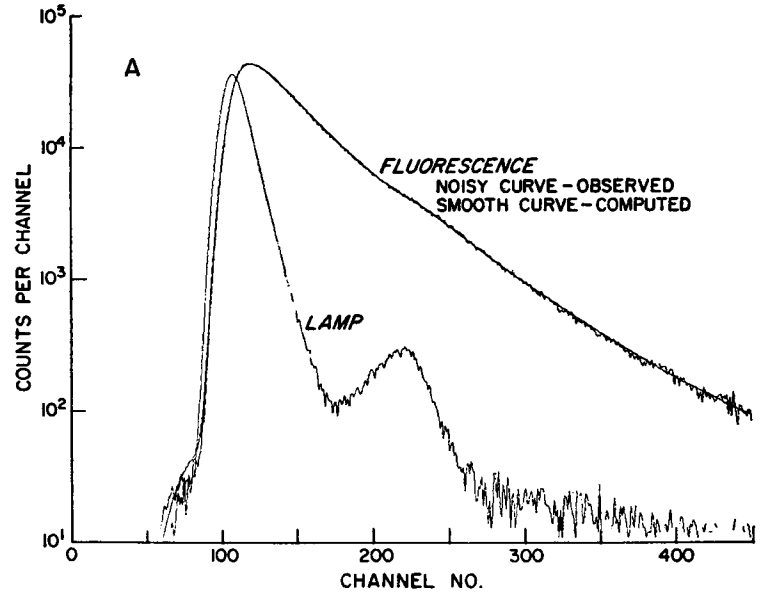


FIGURE 2 Plots of the fluorescence and excitation for the synthetic two component data of Table I, using real lamp data for $E(t)$ in the convolution. Note that both of the calculated curves look good to the eye, but as the data become progressively worse the analyses become less acceptable. One channel = 0.192 ns.



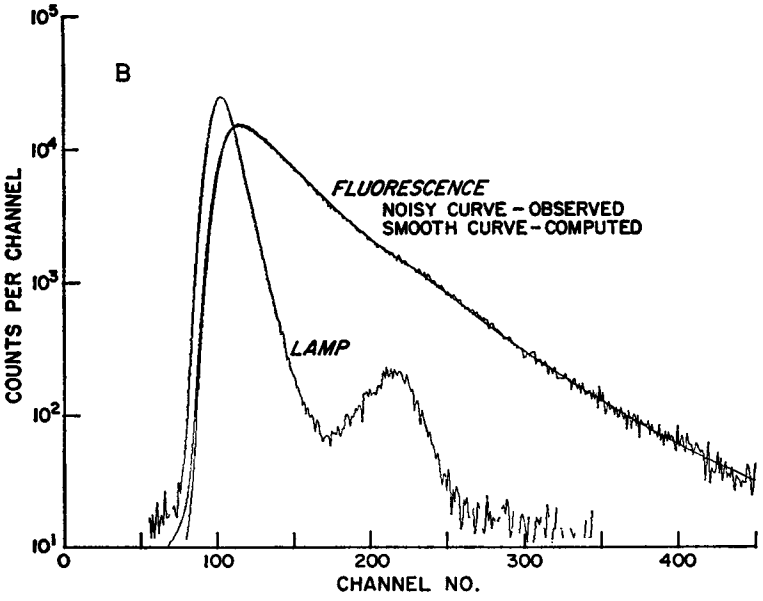


FIGURE 3 The analysis of a mixture of carbazole and anthracene, as an example of a real two-component system with known parameters. Data from Table I. One channel = 0.1805 ns.

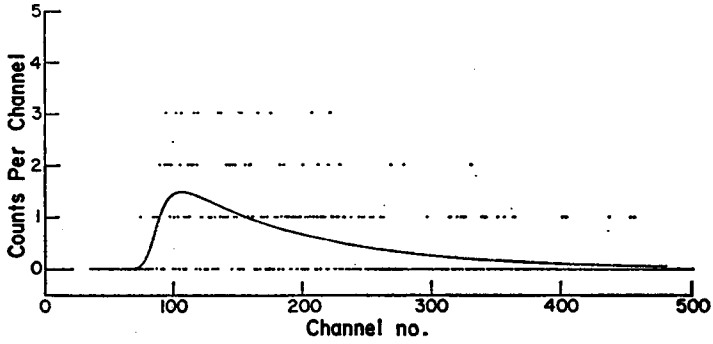


FIGURE 4 Fluorescence curve of quinine bisulfate taken with only 194 counts in 512 channels (see Table I). The points are the fluorescence counts and the solid curve is the computed emission. Analysis gave $\tau = 19.4$ ns with an estimated standard deviation, $\sigma(\tau)$, of 1.3 ns. The figure demonstrates how little data are needed for a good one component analysis. Note that this analysis yielded a decay time that was, by chance, even closer to the correct value than could be expected from the error analysis.

EXAMPLES USING THE COMPONENT INCREMENTATION TEST

Table II shows the use of successive incrementation in judging the number of components in decay data. The rule is very simple: the data are analyzed for 1, 2, 3, $\cdots$ etc. components until an analysis for $N + 1$ components yields the same parameters as an analysis for N components, plus an additional component with a negligibly small amplitude. The negligible component may have either positive or negative amplitudes or time constants. For the cases labeled *a*, *b*, *c*, and *d* in Table II the procedure works. For case *e* the precision of the data is too low for the method to work. Although the analysis is actually not too bad, had there been no knowledge of the parameters, one would not be able to have confidence in the results, at least by the test given. This may be contrasted with case *d*, in which the precision is great enough for component incrementation to be very effective.

EXAMPLES ILLUSTRATING THE USE OF EXPONENTIAL DEPRESSION

Tables III and IV give examples of exponential depression. The number in the column $F_\lambda(t)/F(t)$ is the factor by which the fluorescence value in the last channel of our pulse height analyzer is diminished. Exponential depression lowers the number of iterative loops needed for a self-consistent cutoff correction. The decrease in the number of loops is marked for a two-component analysis; it is dramatic for a three-component analysis. In Table IV, we attempted a component incrementation test by going from a two- to a three-component analysis. The usual analysis did not converge even at 500 loops, but, with an exponential depression in which $F_\lambda(T) = 0.01\ F(T)$, only 6 loops were needed for convergence to the correct parameters.

TABLE II

EXAMPLES OF THE COMPONENT INCREMENTATION TEST*

Data	Preset parameters or experimental conditions	Total counts	One component				Two components	
			α	$\sigma(\alpha)$	τ	$\sigma(\tau)$	α	τ
					ns			*ns*
a	Synthetic data $\alpha = 0.015$ $\tau = 19.4$ ns	268,650	0.015	0.00003	19.37	0.04	0.015 2×10^{-10}	19.37 −272.3
b	Quinine bisulfate 1×10^{-4} M in 0.1 M $(NH_4)_2SO_4$	178,677	0.0099	0.00002	19.37	0.05	0.0099 1.68×10^{-5}	19.32 −14.2

			One component		Two components				Three components	
			α	τ	α	$\sigma(\alpha)$	τ	$\sigma(\tau)$	α	τ
				ns			*ns*			*ns*
c	Synthetic data $\alpha = 0.100$ $\tau = 15.2$ ns $\alpha = 0.200$ $\tau = 7.4$ ns	2,808,326	0.268	11.17	0.101 0.199	0.0021 0.0019	15.18 7.37	0.07 0.06	0.100 0.200 -2×10^{-12}	15.2 7.39 −1302
d	Synthetic data $\alpha = 0.200$ $\tau = 9.8$ ns $\alpha = 0.400$ $\tau = 4.0$ ns	3,345,533	0.496	7.17	0.195 0.405	0.0033 0.0025	9.87 4.04	0.04 0.04	0.194 0.405 -1.5×10^{-5}	9.88 4.05 28.4
e	Synthetic data $\alpha = 0.070$ $\tau = 9.8$ ns $\alpha = 0.090$ $\tau = 4.0$ ns	983,152	0.134	7.81	0.066 0.093	0.0020 0.0016	9.98 4.21	0.08 0.10	0.084 0.075 0.0038	8.83 3.36 14.99

* Note that if N components are present, an analysis for $N + 1$ components leads to a small α and either a positive or negative τ for the extra component. A statistical analysis for $N + 1$ components is not meaningful and hence is not shown.

CONCLUSIONS

Although the method of moments as described earlier (20) has already shown itself, in several ways, to be a practical formalism for handling fluorescence data, the modifications described here further enhance its usefulness. In particular, we present a statistical test for the reliability of the amplitudes and decay constants obtained by the method of moments, and show that the experimental precision, which varies with the number of counts in a monophoton system, agrees with the statistical predictions. In addition, we show that component incrementation can be used to establish the actual number of components present, and that exponential depression may drastically reduce the amount of calculation needed to achieve an analysis while at the same time, in certain cases, improve the accuracy of the parameters obtained.

TABLE III

EXAMPLES OF EXPONENTIAL DEPRESSION*

DATA	$F_\lambda(T)/F(T)$	λ	Analyzed parameters								Loops
			α_1	$\sigma(\alpha_1)$	τ_1	$\sigma(\tau_1)$	α_2	$\sigma(\alpha_2)$	τ_2	$\sigma(\tau_2)$	
					ns				*ns*		
Synthetic data	1	0	0.175	0.0029	9.78	0.041	0.349	0.0021	4.01	0.038	9
α = 0.175	0.93	0.0008	0.175	0.0028	9.78	0.040	0.349	0.0021	4.01	0.036	9
τ = 9.80 ns	0.87	0.0016	0.175	0.0027	9.78	0.040	0.349	0.0021	4.01	0.036	8
α = 0.350	0.75	0.0032	0.175	0.0026	9.78	0.036	0.349	0.0020	4.01	0.033	8
τ = 4.00 ns	0.56	0.0064	0.175	0.0025	9.78	0.036	0.349	0.0019	4.01	0.033	7
2,928,173 counts	0.32	0.0125	0.176	0.0025	9.77	0.034	0.348	0.0019	4.00	0.035	7
	0.10	0.0250	0.176	0.0039	9.77	0.049	0.348	0.0025	4.00	0.057	5
	0.01	0.0510	0.177	0.0110	9.75	0.158	0.347	0.0080	3.99	0.139	3
Synthetic data	1	0	0.103	0.0019	19.24	0.082	0.197	0.0017	9.50	0.068	127
α = 0.100	0.56	0.0064	0.102	0.0016	19.30	0.066	0.198	0.0014	9.54	0.052	84
τ = 19.4 ns	0.32	0.0128	0.101	0.0018	19.34	0.072	0.199	0.0016	9.56	0.063	58
α = 0.200	0.10	0.0256	0.100	0.0041	19.40	0.176	0.199	0.0037	0.60	0.137	31
τ = 9.6 ns	0.01	0.0510	0.099	0.0176	19.48	0.922	0.201	0.0166	9.62	0.475	13
3,592,328 counts											
Real data	1	0	0.0303	0.00075	9.87	0.060	0.0710	0.00062	4.47	0.040	13
2.7×10^{-4} M	0.87	0.0017	0.0304	0.00071	9.86	0.057	0.0709	0.00060	4.46	0.040	12
carbazole	0.75	0.0035	0.0305	0.00069	9.85	0.054	0.0708	0.00058	4.45	0.038	12
4.7×10^{-5} M	0.56	0.0070	0.0307	0.00065	9.84	0.051	0.0707	0.00055	4.44	0.037	11
anthracene	0.32	0.0140	0.0311	0.00067	9.81	0.049	0.0704	0.00056	4.42	0.040	9
2,564,052 counts	0.10	0.0280	0.0315	0.00102	9.77	0.068	0.0700	0.00080	4.40	0.063	7
See Table I	0.01	0.0560	0.0309	0.00358	9.83	0.286	0.0706	0.00297	4.43	0.188	4
	0.0001	0.1120	0.0194	0.01468	11.43	2.866	0.0813	0.01345	4.87	0.603	3

* With increased depression the number of loops for convergence is reduced; the error goes through a minimum.

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APPENDIX I

With G_s defined by Eq. 4, the decay times τ_i are the roots of the polynomial equation

$$\begin{vmatrix} 1 & \tau & \tau^2 & \cdots & \tau^N \\ G_1 & G_2 & G_3 & \cdots & G_{N+1} \\ G_2 & G_3 & G_4 & \cdots & G_{N+2} \\ \vdots & & & \cdots & \\ G_N & G_{N+1} & G_{N+2} & \cdots & G_{2N} \end{vmatrix} = 0. \qquad (A\,1)$$

TABLE IV

EXAMPLE SHOWING A RAPID CONVERGENCE WITH EXPONENTIAL DEPRESSION*

Data	Two components	Three components						
		α_1	τ_1	α_2	τ_2	α_3	τ_3	Loop
Carbazole and anthracene mixture from Table III	$\alpha_1 = 0.030$ $\sigma(\alpha_1) = 0.00075$ $\tau_1 = 9.87$ $\sigma(\tau_1) = 0.06$ $\alpha_2 = 0.071$ $\sigma(\alpha_2) = 0.00062$ $\tau_2 = 4.47$ $\sigma(\tau_2) = 0.04$	0.037	8.79	0.063	4.13	0.0023	13.83	50
		0.037	9.03	0.064	4.16	0.00089	16.3	100
		0.037	9.19	0.065	4.18	0.00029	21.1	300
		0.037	9.24	0.065	4.19	0.00020	23.6	500‡
		Exponential depression (convergence after six loops)						
$F\lambda(T)/F(T) = 0.01$		0.032	9.72	0.070	4.36	3.4×10^{-6}	−5.77	6

* Upon component incrementation without depression no convergence was obtained at 500 loops.

‡ No convergence.

Proof: consider

$$\begin{vmatrix} 1 & \tau & \tau^2 & \cdots & \tau^N \\ 1 & \tau_1 & \tau_1^2 & \cdots & \tau_1^N \\ 1 & \tau_2 & \tau_2^N & \cdots & \tau_2^N \\ \vdots & & & \cdots & \vdots \\ 1 & \tau_N & \tau_N^2 & \cdots & \tau_N^N \end{vmatrix} = 0. \qquad (A\,2)$$

The roots are evidently $\tau_1, \tau_2, \cdots \tau_N$ because any such value makes two rows of the determinant equal. Multiply Eq. A 2 by

$$\begin{vmatrix} 1 & 0 & 0 & \cdots & 0 \\ 0 & \alpha_1\tau_1 & \alpha_2\tau_2 & \cdots & \alpha_N\tau_N \\ 0 & \alpha_1\tau_1^2 & \alpha_2\tau_2^2 & \cdots & \alpha_N\tau_N^2 \\ \vdots & & & & \\ 0 & \alpha_1\tau_1^N & \alpha_2\tau_2^N & \cdots & \alpha_N\tau_N^N \end{vmatrix}, \qquad (A\,3)$$

and Eq. A 1 results.

The inversion of Eq. A 1 arises in other areas as well. For example, the well-known Prony procedure (27, 28) for obtaining the parameters of a sum of exponentials can be put into a formalism identical with that of eq. 6.

The object of Prony's procedure is to determine the amplitudes α_i and the quantities b_i

from a set of data represented by $g(x)$ where

$$g(x) = \sum_{i=1}^{N} \alpha_i \exp\ (b_i x). \tag{A 4}$$

Let

$$y_i = \exp\ (b_i). \tag{A 5}$$

We then have

$$g(x) = \sum_{i=1}^{N} \alpha_i y_i^x. \tag{A 6}$$

We choose data at equally spaced points $x = 1, 2, \cdots N$ and obtain

$$\begin{aligned} g(1) &= \sum_{i=1}^{N} \alpha_i y_i, \\ g(2) &= \sum_{i=1}^{N} \alpha_i y_i^2, \\ g(3) &= \sum_{i=1}^{N} \alpha_i y_i^3, \\ &\vdots \qquad \vdots \\ g(2N) &= \sum_{i=1}^{N} \alpha_i y_i^{2N}. \end{aligned} \tag{A 7}$$

The set (Eq. A 7) is formally identical with Eq. 6. Therefore the set $y_1, y_2, \cdots y_N$ may be found as the roots of

$$\begin{vmatrix} 1 & y & y^2 & \cdots & y^N \\ g(1) & g(2) & g(3) & \cdots & g(N+1) \\ g(2) & g(3) & g(4) & \cdots & g(N+2) \\ \vdots & \vdots & \vdots & \cdots & \vdots \\ g(N) & g(N+1) & & \cdots & g(2N) \end{vmatrix} = 0.$$

APPENDIX II

Some Practical Considerations in Applying the Method of Moments

Some of the more practical aspects of applying the method of moments will be described by outlining the features of a digital computer program that we call FLORTRAN.[1] The program is designed to take advantage of a time-shared or dedicated computer, but it can be readily adapted for batch processing, even on computers of modest size. The comments are intended to describe the pitfalls that are apt to be encountered by those who wish to develop their own computer program or extensively modify ours.

[1] Listings of the program, written in FORTRAN, are available by request to Dr. Isenber or Dr. Dyson.

FLORTRAN provides flexibility by breaking the computations into a number of modules, separately accessible by the user in any order. A particular aspect of the calculation is initiated by typing the appropriate code word onto the teletypewriter console. The code words, and the way in which they are recognized, are given in Table V. A brief description follows.

INPUT causes the experimental data to be read, corrects for background (base line), and calculates moments according to Eqs. 3 *b* and 8. With the monophoton technique, each observed value of excitation and fluorescence, E_j and F_j, is actually the accumulation of counts over a time interval δt, centered about t_j. Thus, for the jth channel

$$E_j = \int_{t_j - \delta t/2}^{t_j + \delta t/2} E(t)\mathrm{d}t, \qquad (A\,8)$$

$$F_j = \int_{t_j - \delta t/2}^{t_j + \delta t/2} F(t)\mathrm{d}t. \qquad (A\,9)$$

Note that each value is δt times the mean of E or F within the channel. However, we have found little error in treating E_j and F_j as if they were δt times $E(t_j)$ and $F(t_j)$, respectively. With that assumption, the moments are calculated by numerical quadrature, using Simpson's formula.

The moments are scaled according to the following equations.

$$m_k^s \equiv m_k/(\tau^k) \qquad (A\,10)$$

$$\mu_k^s \equiv \mu_k/(\alpha\tau^{k+1}). \qquad (A\,11)$$

The parameters α and τ are the amplitudes and time constants of an approximate one-component fit, obtained from Eqs. 4 and 5 without a cutoff correction. The use of m_k^s and μ_k^s leads to G_s (from Eqs. 5) that are scaled to be near unity. The objective is to minimize loss of significant digits in subsequent steps.

We emphasize the importance of using units for E, F, and f that are consistent with the convolution, Eq. 1. We have chosen to express E in counts per unit time and F in counts, making f dimensionless. In transferring data from a pulse height analyzer to a computer, for

TABLE V

SAMPLE CONTROL PROGRAM*

```
      PROGRAM FLORTRAN
    5 PRINT 10
   10 FORMAT(//16H ENTER CODE WORD)
      READ 20, CODE
   20 FORMAT (A6)
      IF(CODE.EQ.6HLOOP    )CALL ITER8
      IF(CODE.EQ.6HADD     )CALL ADD
      IF(CODE.EQ.6HINPUT   )CALL INPUT
      IF(CODE.EQ.6HSETUP   )CALL SETUP
      IF(CODE.EQ.6HRMS     )CALL RMS
      IF(CODE.EQ.6HPLOT    )CALL PLOTEF
      IF(CODE.EQ.6HDEPRES)CALL DEPRES
      IF(CODE.EQ.6HEXIT    )CALL EXIT
      GO TO 5
      END
```

* See Appendix II for explanation.

example, the values of E must be divided by the channel width δt. In practice we perform the moment integration, Eq. 3 *b*, using the original counts and then divided the apparent values of m_k by δt.

SETUP allows the user to preset values of alpha and/or tau.

LOOP initiates the looping needed to provide a cutoff correction (Eq. 8) and prints the (unscaled) values of α_i and τ_i at convergence. Convergence is presumed when the loop-to-loop correction of each α_i and τ_i is less than some preestablished value, usually 0.01%. The τ_i's are obtained by applying standard formulas for polynomial roots to Eq. 6, after expanding it in minors of the first row. Using these values of τ_i, the first N equations of Eq. 4 *b* (i.e., $s = 1, 2, \cdots, N$) are solved by linear algebra. (One could solve the full, overdetermined set of $2N$ equations, but the procedure we use requires less computer time and relies on the lower, and therefore most accurate, moments.)

ADD increases the number of components in the model by one. We have used several methods for finding a first estimate for a cutoff correction to the new model. The most satisfactory procedure we have found is to use the set of α_i's and τ_i's computed for the previous number of components.

DEPRES applies an exponential depression according to Eqs. 13. Again, the assumption is made that E_j and F_j are actually δt times $E(t_j)$ and $F(t_j)$. In addition, however, we assume that E_j and F_j are constant within time channel j, so that

$$E_{j,\lambda} = E_j \int_{t_j - \delta t/2}^{t_j + \delta t/2} e^{-\lambda t} dt = E_j e^{-\lambda t_j} [(e^{-\lambda \delta t/2} - e^{\lambda \delta t/2})/\lambda], \qquad (A12)$$

and similarly for $F_{j,\lambda}$. Since the quantity in brackets ([ ]) is a constant for a given amount of depression (approximately equal to $-\delta t$), it will not affect the values of G, and can thus be ignored.

RMS finds the individual and root mean square deviations between the observed F_j's and the corresponding values calculated from the current set of α_i's and τ_i's. To calculate values of F_j note that Eqs. 1 and 2 may be combined and rearranged as follows:

$$F(t) = \sum_{i=1}^{N} [\alpha_i \int_0^t E(u) \exp(u/\tau_i)\, du] \exp(-t/\tau_i). \qquad (A13)$$

The approximation $F_j^{calc} \simeq F(t_j)$ is thus readily calculated by numerical integration.

The many calls to an exponentiation subprogram implied by Eqs. 10 and A 13 may be avoided by noting that

$$\exp(t_j/\tau_i) = \exp(t_{j-1}/\tau_i) \cdot \exp(\delta t/\tau_i).$$

This saves a considerable amount of computer time, especially when applied to Eq. 10, as that expression must be recalculated at each iteration.

It should be emphasized that deviation tests play no part in the method of moments, but are used only as a check on the quality of curve fitting produced by an analysis. For example, in a perfect analysis from a monophoton system, where only statistical counting errors are present, the frequency of $(F_j^{calc} - F_j)/(F_j^{calc})^{1/2}$ is described by a gaussian distribution with standard deviation of one for those channels where $F_j^{calc} \gg (F_j^{calc})^{1/2}$.

PLOT is an option that permits a visual display of E, F, F^{calc}, and the deviations. The graphs in this paper were produced by it: Fig. 2 on an off-line plotter, and Fig. 3 on a Hewlett-Packard 7200A (Hewlett-Packard Co., Palo Alto, Calif.) attached to the teletypewriter.

EXIT terminates execution.

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APPENDIX III

STUDIES ON THE ANALYSIS OF FLUORESCENCE DECAY DATA BY THE METHOD OF MOMENTS: STATISTICAL APPENDIX

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Sampling Variation of the Estimated Fluorescence Moments

The deviation of the experimental fluorescent intensity curve from the theoretical intensity curve $F_\lambda(t)$ is said to be counting error. This deviation is a consequence of the fact that we observe a finite number of counts sampled from the theoretical population. Here we give an

analytical description of the effect of the sampling error of the counts on the estimated decay parameters.

For a multichannel analyzer with K channels, each of width W, the theoretical relative frequency of counts in the jth channel is given by the normalized area under $F_\lambda(t)$, for $(j-1)\,W < t < jW, j = 1, 2, \cdots K$. The joint behavior of F_j, the number of counts in the jth channel, is given by the multinomial probability function. This probability model refers to conceptual repetitions of the experiment, each with a prescribed total number of counts.

The moments of the depressed fluorescence and lamp intensity functions satisfy the linear system of equations

$$\sum_{j=1}^{k} \frac{m_{\lambda(k-j)}}{(k-j)!}\, G_{\lambda j} = \frac{\mu_{\lambda(k-1)}}{(k-1)!}, k = 1,2,\cdots \tag{A 14}$$

where

$$G_{\lambda j} = \sum_{n=1}^{N} \alpha_n \cdot \theta_n{}^{j}; \tag{A 15}$$

θ_n being the depressed decay time constant

$$\theta_n = \frac{\tau_n}{1+\lambda\cdot\tau_n}. \tag{A 16}$$

The sample estimate of $\mu_{\lambda k}$, the kth order moment of the depressed fluorescence intensity function, may be written as

$$\hat{\mu}_{\lambda k} = W^{k+1} \sum_{i=1}^{K} (i - \tfrac{1}{2})^k \cdot F_{\lambda i}, \tag{A 17}$$

where $F_{\lambda i}$ is the number of depressed counts recorded in the ith channel, i.e.,

$$F_{\lambda i} = e^{-(i-1/2)\lambda W} \cdot F_i. \tag{A 18}$$

Well-known moment properties of the multinomial distribution (1) may be used to derive expressions for the sampling variation of the estimated fluorescence moments.

It may be shown that the estimated variances and covariance are given by

$$\begin{aligned} \delta^2[\hat{\mu}_{\lambda k}] &= W\cdot \left[\hat{\mu}_{2\lambda(2k)} - \frac{\hat{\mu}^2_{\lambda k}}{\mu'_{00}}\right], \\ \operatorname{cov}[\hat{\mu}_{\lambda l}, \hat{\mu}_{\lambda k}] &= W\cdot \left[\hat{\mu}_{2\lambda(k+l)} - \frac{\hat{\mu}_{\lambda k}\cdot\hat{\mu}_{\lambda l}}{\mu'_{00}}\right], \end{aligned} \tag{A19}$$

respectively, where μ'_{00} is the nondepressed 0th-order moment [$\mu'_{00} = W\cdot$ (Total number of counts)]. We now show how these sampling properties of the estimated fluorescence moments are used to derive approximate standard errors for the estimated decay parameters.

Only for the case of a small number (say, $N \leq 2$) of exponential components is it feasible to write explicit expressions giving the estimates $\hat{\alpha}_n$, $\hat{\theta}_n$ as functions of the estimated moments $\hat{\mu}_{\lambda k}$, $k = 1, 2, \cdots, 2N - 1$. For general N the method of moments does however define $\hat{\alpha}_n$ and $\hat{\theta}_n$ as implicit functions of $\hat{\mu}_{\lambda k}$, which is sufficient for the development that follows. Approximations to the standard errors of $\hat{\alpha}_n$ and $\hat{\theta}_n$ may be developed by considering the linear terms of a Taylor series expansion of $\hat{\theta}_n$ and $\hat{\alpha}_n$ around $\mu_{\lambda k}$, $k = 1, 2, \cdots, 2N - 1$ (reference 2).

Taking the variances of these linear approximations, and employing the estimated variance and covariance of $\hat{\mu}_{\lambda k}$, as given by Eq. A 19, we arrive at the following expressions for the approximate variances of the estimated decay parameters

$$\hat{\sigma}^2(\hat{\theta}_n) \doteq W \cdot \sum_{j=1}^{2N-1} \left(\frac{\partial \hat{\theta}_n}{\partial \hat{\mu}_{\lambda j}}\right)^2 \left[\hat{\mu}_{2\lambda(2j)} - \frac{\hat{\mu}^2_{\lambda j}}{\mu'_{00}}\right] + 2W \sum_{l>j} \sum \left(\frac{\partial \hat{\theta}_n}{\partial \hat{\mu}_{\lambda j}}\right)\left(\frac{\partial \hat{\theta}_n}{\partial \hat{\mu}_{\lambda j}}\right)\left[\hat{\mu}_{2\lambda(j+l)} - \frac{\hat{\mu}_{\lambda j} \cdot \hat{\mu}_{\lambda l}}{\mu'_{00}}\right], \quad \text{(A 20)}$$

$$\hat{\sigma}^2(\hat{\alpha}_n) \doteq W \cdot \sum_{j=1}^{2N-1} \left(\frac{\partial \hat{\alpha}_n}{\partial \hat{\mu}_{\lambda j}}\right)^2 \left[\hat{\mu}_{2\lambda(2j)} - \frac{\hat{\mu}^2_{\lambda j}}{\mu'_{00}}\right] + 2W \cdot \sum_{l>j} \sum \left(\frac{\partial \hat{\alpha}_n}{\partial \hat{\mu}_{\lambda l}}\right)\left(\frac{\partial \hat{\alpha}_n}{\partial \hat{\mu}_{\lambda j}}\right)\left[\hat{\mu}_{2\lambda(j+l)} - \frac{\hat{\mu}_{\lambda j} \cdot \hat{\mu}_{\lambda l}}{\mu'_{00}}\right]. \quad \text{(A 21)}$$

Our final consideration is the method of evaluation of the partial derivatives appearing in Eqs. A 20 and A 21. For convenience of notation, the circumflex indicating an estimated quantity and the lambda subscript will be suppressed in what follows.

Using the chain rule of differentiation, we may write

$$(\partial \theta_n / \partial \mu_j) = \sum_{i=1}^{2N} (\partial \theta_n / \partial G_i)(\partial G_i / \partial \mu_j).$$

$n = 1, 2, \cdots, N; j = 1, 2, \cdots, 2N - 1$. From Eq. A 14 we see that $\partial G_i / \partial \mu_j = 0$ for $i \leq j$, so that we have

$$(\partial \theta_n / \partial \mu_j) = \sum_{i=j+1}^{2N} (\partial \theta_n / \partial G_i)(\partial G_i / \partial \mu_j). \quad \text{(A 22)}$$

The solutions G_i of the linear system of equations A 14 may be expressed as ratios of determinants, the denominator being the determinant of coefficients which is easily seen to be m_0^{2N}.

Partial differentiation of the numerator determinant with respect to μ_j, and simplification leads to the following expressions for $\partial G_i / \partial \mu_j$. For $i = j + 1$,

$$\frac{\partial G_i}{\partial \mu_j} = \frac{1}{j! m_o}. \quad \text{(A 23)}$$

For $j = 1, 2, \cdots, 2N-1; j+2 \leq i \leq 2N$,

$$\frac{\partial G_i}{\partial \mu_j} = \frac{(-1)^{(i+j+1)}}{j!\, m_0^{(i-j)}} \begin{vmatrix} m_1 & m_0 & 0 & 0 & \cdots & 0 \\ \frac{m_2}{2!} & m_1 & m_0 & 0 & \cdots & 0 \\ \frac{m_3}{3!} & \frac{m_2}{2!} & m_1 & m_0 & 0 \cdots & 0 \\ \cdot & \cdot & & & & \cdot \\ \cdot & \cdot & & & & \cdot \\ \cdot & \cdot & & & & 0 \\ \frac{m_{i-j-1}}{(i-j-1)!} & & & & \cdots & m_0 \\ & & & & & m_1 \end{vmatrix}. \tag{A 24}$$

These general expressions which hold for any number N of exponential components will be evaluated for the special cases of $N = 1$ and 2 in the following section.

In order to evaluate the other partial derivatives, $\partial\theta_n/\partial G_i$, which are required in Eq. A 22, we turn to the determinential equation whose roots are the values of the estimated transformed decay constants, $\theta_1, \theta_2, \cdots, \theta_N$

$$|\Delta| = \begin{vmatrix} 1 & \theta & \theta^2 & \cdots & \theta^N \\ G_1 & G_2 & \cdots & & G_{N+1} \\ G_2 & G_3 & \cdots & & G_{N+2} \\ \cdot & \cdot & & & \cdot \\ \cdot & \cdot & & & \cdot \\ \cdot & \cdot & & & \cdot \\ G_N & G_{N+1} & \cdots & & G_{2N} \end{vmatrix} = 0. \tag{A 25}$$

Denoting the cofactor of the $(1, j)$ element of the matrix Δ by C_{1j}, we may write eq. A 25 as

$$\sum_{j=1}^{N+1} C_{1j}\theta^{j-1} = 0. \tag{A 26}$$

This Nth-degree polynomial in θ determines the roots θ_n, $n = 1, 2, \cdots, N$ as implicit functions of the G_i, $i = 1, 2, \cdots, 2N$. Partial differentiation of Eq. A 26 with respect to G_i yields

$$\frac{\partial\theta}{\partial G_1} = \frac{-\sum_{j=1}^{N+1} (\partial C_{1j}/\partial G_i)\theta^{(j-1)}}{\sum_{j=1}^{N+1} (j-1)C_{1j}\theta^{(j-2)}} \tag{A 27}$$

for $\theta = \theta_1, \theta_2, \cdots, \theta_N$, and $i = 1, 2, \cdots, 2N$. This completes the description of the general scheme for calculating the partial derivatives $\partial\theta_n/\partial\mu_j$, which are then substituted into Eq. A 20, allowing us to calculate $\hat{\sigma}^2(\hat{\theta}_n)$.

In order to calculate the variances of $\hat{\alpha}_n$, $n = 1, 2, \cdots, N$ from Eq. A 21, we must first evaluate the partial derivatives $\partial\alpha_n/\partial\mu_j$. Recalling the definition

$$G_{\lambda i} = \sum_{n=1}^{N} \alpha_n \theta_n^i,$$

we see that

$$\partial G_i/\partial \mu_j = \sum_{n=1}^{N} (\partial \alpha_n/\partial \mu_j)\theta_n^i + i \sum_{n=1}^{N} \alpha_n \theta_n^{i-1}(\partial \theta_n/\partial \mu_j). \qquad (A\ 28)$$

We have already evaluated the derivatives $\partial G_i/\partial \mu_j$, $\partial \theta_n/\partial \mu_j$, and have available the estimates of α_n and θ_n. Hence the system of Eq. A 28 for fixed j, where $i = 1, 2, \cdots, N$, consists of N linear nonhomogeneous equations in the N unknowns $\partial \alpha_n/\partial \mu_j$, which are easily solved by the standard determinant method.

We have thus given a procedure for evaluating the approximate variances of $\hat{\alpha}_n$ and $\hat{\theta}_n$, for any number of exponential components. The estimates of the intrinsic decay time parameters τ_n are obviously $\hat{\tau}_n = \hat{\theta}_n/(1 - \lambda \cdot \hat{\theta}_n)$; from which the approximate variances may be seen to be

$$\hat{\sigma}^2(\hat{\tau}_n) \doteq \frac{\hat{\sigma}^2(\hat{\theta}_n)}{(1 - \lambda \cdot \hat{\theta}_n)^4}, \quad n = 1, 2, \cdots, N. \qquad (A\ 29)$$

In the numerical examples presented in the experimental paper the precision of estimation is expressed in terms of the approximate standard errors $[\hat{\sigma}^2(\hat{\tau}_n)]^{1/2}$, $[\hat{\sigma}^2(\hat{\alpha}_n)]^{1/2}$.

Explicit Expressions for One- and Two-Component Systems

For a one-component system ($N = 1$), the system of Eqs. A 14 becomes

$$m_0 G_1 = \mu_0,$$

$$m_1 G_1 + m_0 G_2 = \mu_1,$$

which solves to give

$$G_1 = \frac{\mu_0}{m_0}, \quad G_2 = \frac{m_0\mu_1 - m_1\mu_0}{m_0^2}.$$

Substitution into Eq. A 25 then yields $\theta_1 = (\mu_1/\mu_0) - (m_1/m_0)$, which together with the relationship $G_1 = \alpha_1\theta_1$, gives

$$\alpha_1 = \frac{\mu_0^2}{m_0\mu_1 - m_1\mu_0}.$$

Evaluation of the required partial derivatives and substitution into Eqs. A 20 and A 21 results in the following expressions for the approximate variances

$$\hat{\sigma}^2(\hat{\theta}_1) = \frac{W}{\mu_{\lambda 0}^2} \cdot \left[\hat{\mu}_{(2\lambda)2} - \frac{\mu_{\lambda 1}^2}{\mu'_{00}}\right]$$

$$\hat{\sigma}^2(\hat{\alpha}_1) = \frac{m_{\lambda 0}^2 \mu_{\lambda 0}^4 \cdot W}{(m_{\lambda 0} \cdot \mu_{\lambda 1} - m_{\lambda 1}\mu_{\lambda 0})^4} \cdot \left[\mu_{(2\lambda)2} - \frac{\mu_{\lambda 1}^2}{\mu'_{00}}\right]. \qquad (A\ 30)$$

For a two-component system, the determinantal Eq. A 25 becomes

$$\begin{vmatrix} 1 & \theta & \theta^2 \\ G_1 & G_2 & G_3 \\ G_2 & G_3 & G_4 \end{vmatrix} = 0.$$

Differentiating the cofactors C_{1j}, $j = 1, 2, 3$, with respect to G_i, $i = 1, 2, 3, 4$, substituting into Eq. A 27, and expressing C_{1j} and G_i in terms of α_n and θ_n, we obtain

$$\begin{aligned} \frac{\partial\theta_1}{\partial G_4} &= \frac{1}{\alpha_1\theta_1(\theta_1-\theta_2)^2}, & \frac{\partial\theta_2}{\partial G_4} &= \frac{1}{\alpha_2\theta_2(\theta_1-\theta_2)^2}, \\ \frac{\partial\theta_1}{\partial G_3} &= \frac{-(\theta_1+2\theta_2)}{\alpha_1\theta_1(\theta_1-\theta_2)^2}, & \frac{\partial\theta_2}{\partial G_3} &= \frac{-(\theta_2+2\theta_1)}{\alpha_2\theta_2(\theta_1-\theta_2)^2}, \\ \frac{\partial\theta_1}{\partial G_2} &= \frac{\theta_2(\theta_2+2\theta_1)}{\alpha_1\theta_1(\theta_1-\theta_2)^2}, & \frac{\partial\theta_2}{\partial G_2} &= \frac{\theta_1(\theta_1+2\theta_2)}{\alpha_2\theta_2(\theta_1-\theta_2)^2}. \end{aligned} \tag{A 31}$$

From the general results given by Eqs. A 23 and A 24 we see that

$$\begin{aligned} \frac{\partial G_4}{\partial\mu_3} &= \frac{1}{6m_0}, & \frac{\partial G_3}{\partial\mu_2} &= \frac{1}{2m_0}, & \frac{\partial G_2}{\partial\mu_1} &= \frac{1}{m_0}, \\ \frac{\partial G_4}{\partial\mu_1} &= \frac{-m_1}{2m_0^2}, & \frac{\partial G_3}{\partial\mu_1} &= \frac{-m_1}{m_0^2}, \\ \frac{\partial G_4}{\partial\mu_1} &= \frac{2m_1^2-m_0m_2}{2m_0^3}. \end{aligned} \tag{A 32}$$

The partial derivatives given by Eqs. A 31 and A 32 are used in Eq. A 22 to evaluate $\partial\theta_n/\partial\mu_j$, which are then substituted into expression A 22, completing the calculation of $\delta^2(\hat{\theta}_1)$, $\delta^2(\hat{\theta}_2)$. Finally, employing Eq. A 28, we obtain

$$\begin{aligned} \frac{\partial\alpha_1}{\partial\mu_1} &= \frac{\{1-m_0[2\alpha_1\theta_1(\partial\theta_1/\partial\mu_1)+2\alpha_2\theta_2(\partial\theta_2/\partial\mu_1)]\}+\theta_2m_0[\alpha_1(\partial\theta_1/\partial\mu_1)+\alpha_2(\partial\theta_2/\partial\mu_1)]}{m_0\theta_1(\theta_1-\theta_2)}, \\ \frac{\partial\alpha_2}{\partial\mu_1} &= \frac{\{1-m_0[2\alpha_1\theta_1(\partial\theta_1/\partial\mu_1)+2\alpha_2\theta_2(\partial\theta_2/\partial\mu_1)]\}+\theta_1m_0[\alpha_1(\partial\theta_1/\partial\mu_1)+\alpha_2(\partial\theta_2/\partial\mu_1)]}{m_0\theta_2(\theta_2-\theta_1)}, \\ \frac{\partial\alpha_1}{\partial\mu_2} &= \frac{\theta_2[\alpha_1(\partial\theta_1/\partial\mu_2)+\alpha_2(\partial\theta_2/\partial\mu_2)]-[2\alpha_1\theta_1(\partial\theta_1/\partial\mu_2)+2\alpha_2\theta_2(\partial\theta_2/\partial\mu_2)]}{\theta_1(\theta_1-\theta_2)}, \\ \frac{\partial\alpha_2}{\partial\mu_2} &= \frac{\theta_1[\alpha_1(\partial\theta_1/\partial\mu_2)+\alpha_2(\partial\theta_2/\partial\mu_2)]-[2\alpha_1\theta_1(\partial\theta_1/\partial\mu_2)+2\alpha_2\theta_2(\partial\theta_2/\partial\mu_2)]}{\theta_2(\theta_2-\theta_1)}, \\ \frac{\partial\alpha_1}{\partial\mu_3} &= \frac{\theta_2[\alpha_1(\partial\theta_1/\partial\mu_3)+\alpha_2(\partial\theta_2/\partial\mu_3)]-[2\alpha_1\theta_1(\partial\theta_1/\partial\mu_3)+2\alpha_2\theta_2(\partial\theta_2/\partial\mu_3)]}{\theta_1(\theta_1-\theta_2)}, \\ \frac{\partial\alpha_2}{\partial\mu_3} &= \frac{\theta_1[\alpha_1(\partial\theta_1/\partial\mu_3)+\alpha_2(\partial\theta_2/\partial\mu_3)]-[2\alpha_1\theta_1(\partial\theta_1/\partial\mu_3)+2\alpha_2\theta_2(\partial\theta_2/\partial\mu_3)]}{\theta_2(\theta_2-\theta_1)}. \end{aligned} \tag{A 33}$$

Substitution of these partials into Eq. A 21 completes the calculation of $\delta^2(\hat{\alpha}_1)$, $\delta^2(\hat{\alpha}_2)$.

Examples of the procedure developed here are given in the experimental part of the paper. These show that our results are in good agreement with experimental analysis, both synthetic and real. This demonstrates the usefulness of the statistical results. It also demonstrates the adequacy of our approximate treatment of the problem.

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