THE ANALYSIS OF FLUORESCENCE DECAY BY A METHOD OF MOMENTS

IRVIN ISENBERG *and* ROBERT D. DYSON

From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331

ABSTRACT The fluorescence decay of the excited state of most biopolymers, and biopolymer conjugates and complexes, is not, in general, a simple exponential. The method of moments is used to establish a means of analyzing such multi-exponential decays. The method is tested by the use of computer simulated data, assuming that the limiting error is determined by noise generated by a pseudorandom number generator. Multi-exponential systems with relatively closely spaced decay constants may be successfully analyzed. The analyses show the requirements, in terms of precision, that data must meet. The results may be used both as an aid in the design of equipment and in the analysis of data subsequently obtained.

INTRODUCTION

Considerable interest exists in fluorescence decay studies of proteins and protein adsorbates and conjugates. Such studies are important for a variety of reasons. They are needed, for example, as adjuncts to the measurement of rotatory diffusion by steady-state fluorescence depolarization methods. However, as Stryer (1) has emphasized, time decay studies have considerably more importance as independent means of examining the anisotropy of emission. By looking at the kinetics of the anisotropy of emission rather than the steady-state polarization, we can determine the rotatory diffusion constants with a minimum of assumptions. For example, in steady-state measurements it is often necessary to use a series of solvents of different viscosities, with the concomitant assumption that the solvent effect is only due to viscosity. With dynamic methods measurements need be made only in the solvent of interest.

Tao (2) has analyzed the decay of the components of fluorescence when the emitter is rigidly bound to a macromolecule undergoing rotatory Brownian diffusion. For the most general shape the anisotropy is a sum of five exponentials. If an axis of symmetry exists, the anisotropy is the sum of three exponents, but only in the case of a sphere is the anisotropy a single exponential. If a protein is flexible so that new degrees of freedom arise, the decay curves are still more complex. In this connection it is interesting to note that Chen (3) has emphasized that flexibility may be the rule rather than the exception for proteins.

Even if one measures the decay of the total intensity and not the anisotropy of emission, the luminescence may still be complex, arising from a heterogeneous population of emitting protein chromophores or, if the protein is tagged with a dye, a heterogeneous population of dye-binding sites. Wahl and Lami (4), for example, reported that the decay of fluorescence of the 1-dimethylaminonaphthalene-5-sulfonyl-lysozyme conjugate is characterized by two exponential decays. Weber (5) has found that bovine serum albumin-anilino-naphthalene sulfonate conjugates have multiple component fluorescence decays, reflecting a heterogeneity in the population of bound ligands. Chen (6) also reports a heterogeneity in the binding of ligands to bovine serum albumin.

It appears as if the decays of perhaps all nucleic acid conjugates, and most proteins, protein conjugates, and protein adsorbates will be complex rather than simple exponentials. One would expect a single exponential decay only for the anisotropy or the total emission from spherical, rigid macromolecules containing one emitter or a set of noninteracting identical emitters. However, as noted by Tao (2), the use of time-dependent methods to study spherical molecules yields little or no information beyond that found by static techniques, apart from a demonstration that a particular molecule is indeed spherical. It thus appears as if most of the interesting data on the time decay of protein and nucleic acid emission will have multicomponent form.

The collection and analysis of multicomponent data has intrinsic difficulties, due mainly to the large number of parameters that must be determined. If n components are present, n decay constants and $n - 1$ ratios of the amplitude of the decays must be found. To determine uniquely even a small number of components therefore requires data with a high degree of precision, and a means of analysis that minimizes the introduction of error. Furthermore, the analysis must not be sensitive to a subjective choice of a model.

There are two broad classes of techniques for obtaining time-decay data–cross correlation methods in which the response to modulated exciting light is measured (7–11) and pulse techniques in which the response to discrete flashes is measured (2, 4, 12–19).

While practical methods for the analysis of data obtained by cross-correlation techniques have been presented (9–11), no generally acceptable procedure exists for the analysis of the response to discrete flashes. Attempts to analyze such data may generally be put into three classes. In the first, which may be called a subtraction procedure, the decay curve is first examined at times sufficiently long so that all but one of the decays has become negligibly small. The slowest decay is thereby determined. The effect of this decay is then subtracted from the data, and the next slowest decay determined. In principle, all components may be determined in this way; in practice, the procedure involves the subtraction of quantities that are nearly equal and the experimental errors become rapidly magnified and quickly dominate the procedure. A second class of methods relies on curve-fitting procedures. Such methods are in principle capable of analyzing decay data, but in practice curve fitting is sensitive to the choice of the number of components to be fitted and extremely sensitive to even small errors in the data. A complex curve which can be approximated by n exponentials can be fit as well by $n + 1$, with the additional one being nontrivial in some sense. We believe that without some additional and independent knowledge of the system, curve-fitting procedures are not of much value in the analysis of multicomponent decays. Such knowledge usually is not available.

A third method, originating with Bay (20), uses the moments of experimental data. This "method of moments" was used by Brody (12) for a one component analysis of fluorescence lifetime, and a combined method of moments and subtraction procedure was used by Wahl and Lami (4). The method of moments recommends itself as a procedure since, among other things, its use of only integrals of the experimental data minimizes the effects of noise.

This paper may be considered as an extension of Bay's (20) work. It provides a general scheme for the analysis of decay data in those cases in which the response to a delta function excitation may be represented as a sum of exponentials. Such cases include that of rotatory diffusion (2) and also that of independently decaying species in a sample. Cases involving other types of decay, such as those arising in problems of energy transfer (21) or those involving biphotonic or bimolecular processes (22), must be handled in a different fashion.

We believe that the method of moments provides a satisfactory scheme for the analysis of decay data of the multi-exponential type. We also believe that it will have applicability in areas other than time decay. It may be useful, for example, in the analysis of equilibrium sedimentation data, where a decomposition into exponentials is necessary.

DERIVATION OF EQUATIONS

Let $f(t)$ be the luminescence intensity from a sample excited by a Dirac delta function flash at $t = 0$. As explained above we consider samples whose emission is the sum of exponentials, and write

$$f(t) = \sum_{n=1}^{N} \alpha_n e^{-\lambda_n t}. \qquad (1)$$

The problem[1] is to determine the number of components[2] N, the amplitudes, α_n, and the decay constants, λ_n.

---

[1] This statement of the problem appears reasonable, but actually is oversimplified. A more sophisticated statement and discussion is given later in the paper.

[2] The term "components" is used in a general sense in this paper. If the total intensity is being analyzed, the number of components is the number of independent species. However if the anisotropy is being investigated the "number of components" or "number of species" means the number of exponentials in the intensity component under discussion. Formally, there is no difference between the two, but it is convenient to discuss the analysis as if one always dealt with independent species.

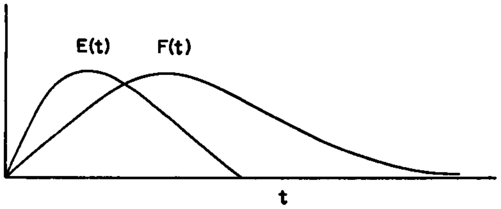

FIGURE 1 Schematic representation of the lamp flash, $E(t)$ and the fluorescence, $F(t)$.

In practice the flash of light used to excite the sample is of finite duration, and may be comparable in lifetime to the fluorescence lifetime of the sample; consequently the exciting light cannot usually be approximated by a delta function.

Let $E(t)$ be the intensity of the exciting lamp and $F(t)$ the emission of the sample when excited by $E(t)$.

$E(t)$ and $F(t)$ are shown, schematically, in Fig. 1.

$F(t)$ is given by the convolution

$$F(t) = \int_0^t E(t-u)f(u)\,du. \qquad (2)$$

$E(t)$ and $F(t)$ constitute, of course, the experimentally determined data.

Let us define the k^{th} moments of F and E by

$$\mu_k = \int_0^\infty t^k F(t)\,dt \qquad (3)$$

and

$$m_k = \int_0^\infty t^k E(t)\,dt. \qquad (4)$$

These moments may now be considered the given experimental information.

Taking the k^{th} moment of F, we obtain

$$\mu_k = \int_0^\infty t^k \int_0^t E(t-u)f(u)\,du\,dt. \qquad (5)$$

The right hand side of equation 5 is the double integral of $t^k E(t-u)f(u)$, in the u, t plane, taken over the area bounded by the lines $u = 0$ and $u = t$. By reversing the order of integration, one obtains

$$\mu_k = \int_0^\infty f(u) \int_0^\infty (u+v)^k E(v)\,dv\,du \qquad (6)$$

$$= k! \sum_{s=1}^{k+1} G_s\,(m_{k+1-s})/(k+1-s)! \qquad (7)$$

where G_s is defined by

$$G_s = \sum_{n=1}^{N} \alpha_n/\lambda_n{}^s \tag{8 a}$$

$$= \sum_{n=1}^{N} \alpha_n \tau_n{}^s. \tag{8 b}$$

For successive values of k, equation 7 provides a set of linear equations,

$$\begin{aligned} \mu_0 &= G_1 m_0 \\ \mu_1 &= G_1 m_1 + G_2 m_0 \\ \mu_2/2! &= G_1 m_2/2! + G_2 m_1 + G_3 m_0 \quad \text{etc.} \end{aligned} \tag{9}$$

These equations may be used to solve for the G's, given the moments μ_i, m_i. A set of $2N$ G's completely characterizes a given sample. In other words a knowledge of such a set is equivalent to a specification of $\alpha_1, \cdots \alpha_N, \lambda_1, \cdots \lambda_N$. For small values of N, the α's and λ's may be determined from $G_1, G_2, \cdots G_{2N}$ by elementary algebra. For larger values of N, Prony's method (23) may be used. Any G_s, for $s > 2N$, must be a function of the first $2N$ values of G_s.

The relationship between the first $2N + 1$ values of G_s is given by the following theorem, proven in Appendix 1.

The necessary and sufficient condition for the system to have N *components is that* N *be the smallest number for which*

$$\Delta_{N+1} \equiv \begin{vmatrix} G_1 & G_2 & \cdots & G_{N+1} \\ G_2 & G_3 & \cdots & G_{N+2} \\ \vdots & & & \\ G_{N+1} & G_{N+2} & \cdots & G_{2N+1} \end{vmatrix} = 0 \quad . \tag{10}$$

If it were possible to obtain ideal, errorless data, equation 10 could be used to obtain N directly from the set G_s. In practice, however, Δ_{N+1} will not vanish identically, and a different criterion must be established for deciding the number of species in the sample. This is discussed below.

It should be emphasized that because of experimental error, it is often impossible to give an absolute meaning to N, the number of components. Suppose, for example, that experimental data is obtained from a system that has but a single decay, with the decay constant λ_1. It will always be possible to satisfy the experimental data by assuming that two components are present, with decay constants $\lambda_1 - \Delta_1$ and $\lambda_1 + \Delta_2$, provided Δ_1 and Δ_2 are small enough. In other words experimental errors preclude the possibility of resolving two components that have sufficiently close decay constants, or even of deciding if two species, rather than one, exist. Similarly, in multicomponent systems, it is impossible, in an absolute sense, to state how many components are present.

CUT-OFF CORRECTION

Unfortunately the emission data, $F(t)$, extends only to some finite time, T, rather than to infinity as required in the above procedure. Approximating the moments of $F(t)$,

$$\mu_k = \int_0^\infty t^k F(t)\, dt$$

by

$$\mu_k \approx \int_0^T t^k F(t)\, dt \tag{11}$$

may introduce significant errors, which become progressively worse as k becomes larger. No such difficulty is encountered with the excitation data, $E(t)$, since it rapidly approaches zero at early values of t.

The cut-off error is important and it appears as if, for reasonable sets of data, it is impossible to use the method of moments for a multicomponent analysis without a good correction. For such a correction, we have adopted an iterative procedure, wherein μ_k is first approximated from the available data, according to equation 11, and then corrected by adding

$$\delta\mu_k = \int_T^\infty t^k \sum_{n=1}^{N} \alpha_n e^{-\lambda_n t}\, dt. \tag{12}$$

To calculate equation 12 approximate values of α_n and λ_n are used. The resulting moments are then used in equation 9 to find new values of α_n and λ_n, which then allow a second estimate of $\delta\mu_k$. The process is repeated until a self-consistent set of values is obtained, i.e., until an arbitrarily small change in each of the parameters is achieved at each iteration.

The convergence of the iteration is followed by finding the root mean square difference between the values of μ_k calculated from equation 11 and estimates (est) based on the equation

$$\mu_k^{\text{est}} = \int_0^T t^k \sum_{n=1}^{N} \alpha_n \int_0^t E(t-\mu) e^{-\lambda_n \mu}\, d\mu\, dt. \tag{13}$$

For rapid convergence we desire $\delta\mu_k$ to be as small as possible. This requires that we use data at long enough times for the fluorescence to decay to low values. However, as the emission becomes smaller, the signal-to-noise ratio increases and the analysis becomes worse. It is therefore not feasible, in general, to make $\delta\mu_k$ arbitrarily small and it is this feature that makes the cut-off correction important.

ANALYSIS BY COMPUTER PROGRAM

To test the procedure we have written programs to generate data, add noise, and then analyze such noisy data by the procedure described here. In this fashion we are able to see how well the method works in terms of requirements of signal to noise. It has turned out, however, that the precision requirements for a good multicomponent analysis exceed what has customarily been obtained by workers in the field.

The programs which we have written were designed for execution from a remote terminal of Oregon State University's time-shared Control Data 3300. The programs were written to allow the operator to follow each step of the calculation as it occurs, and to intercede in order to change the path of execution at numerous points. The main features of the analysis program are outlined below.

1. N moments are calculated from the excitation data, E, and an equal number from the fluorescence data, F, according to equations 4 and 11.
2. An attempt is made to reconcile these moments with a single decay, by fitting the fluorescence curve at long times to a single exponential. The constants, α and λ, for this hypothetical single component are determined from the fit.
3. These values of α and λ are used to calculate a cut-off correction, $\delta\mu_k$, equation 12.
4. From the values of $\delta\mu_k$ and the experimental values of m_k and μ_k, a set of G's is determined from the set of equations 9.
5. The set of G's is then solved for new values of α and λ.
6. These new values are used to calculate moments of F, which are then compared with the experimentally determined moments.
7. If the fit is unsatisfactory, as determined by the operator, control returns to step 3.
8. If the fit is satisfactory, or seems to be as good as it is going to get, the number of components is increased by one and control is shifted to step 3, or execution is terminated.

EXAMPLES OF RESULTS USING COMPUTER SIMULATED DATA

Data was simulated as if it were collected by a monophoton technique (13, 17), using a 400 channel analyzer to collect fluorescence data, and 400 channels to collect exciting lamp data. It was assumed that each channel was equivalent to 0.6 nsec.

We have chosen to present the data in terms of a monophoton technique, since this methodology appears to offer important advantages over other presently used methods in maximizing signal-to-noise ratios. However, it should be emphasized that the data presented have more general implications in showing how the analyses change as the noise is varied.

The exciting lamp was specified to have a flash characteristic of

$$\begin{aligned} N &= 10^5 \sin \pi t/30 \qquad && 0 \leqq t \leqq 30 \\ &= 0 && 30 < t \end{aligned}$$

where N is the number of counts at a time t, in nanoseconds. This simulated lamp has a flash duration longer than that of most lamps commonly used and therefore provides a stringent test of the method.

In a series of trials the number of counts in the 400 channels of fluorescence data was varied from one analysis to another to determine the effects of counting error on the results.

After choosing a set of α_n, λ_n, the convolution, equation 2, was calculated. A pseudorandom number generator added noise to the data of a magnitude that one can expect to observe in practice. The standard deviation was given by the square root of the number of counts in a given channel and a background "dark signal" of 50 counts per channel was assumed.

In this paper we present illustrative examples only. Due to the large number of parameters that may be varied, a definitive elucidation of the range of applicability would be difficult.

It is not always best to use all 400 channels for analysis. At the tail end of the curves, the signal-to-noise ratio becomes poor and we have found that the additional information gained by following the decay to a low number of counts per channel does not offset the problems posed by the greater noise of this region.

ONE COMPONENT DATA

For a sufficiently large number of counts, a one component analysis converges rapidly to the correct value. Table I shows a typical set of results.

In all of the analyses presented in Table I, the iteration procedure stabilized to three or more significant figures after one correction loop. It may be noted that the decay time, 12.0 nsec, is considerably shorter than the lamp flash duration.

It is to be expected that the accuracy of analysis increases as the number of counts rises. However, the values of α and τ approach the correct values rather rapidly, as the number of counts increases through a surprisingly small range. At 6390 counts there is a rather large error, but at 25,600 counts the analyzed parameters are within 1 % of the correct values. We have observed the same phenomenon in all of our analyses. With a small number of counts poor results are obtained, as expected.

TABLE I

DATA SYNTHESIZED FROM $f = 1.00\ e^{-t/12.0}$

Total counts in F	Maximum value of F, in counts	Parameters found	
		α	τ
6.39×10^3	189	0.74	17.0
12.8×10^3	328	0.84	14.6
25.6×10^3	606	1.01	11.9
63.9×10^3	1,440	1.01	12.0
63.9×10^4	13,948	0.99	12.0

The results change as the number of counts is raised. During a relatively small increase in the number of counts, the analyzed results come close to the correct values. Further increases have little effect on the computation accuracy.

TWO COMPONENT DATA

Table II shows the results of a typical two component analysis. The values in Table II stabilized to three or more significant figures after six iterations.

A number of trials have been made to see how small a second component may be and still be detected. We cite only one example. For lifetimes of 12 and 18 nsec, either exponent may have an amplitude 10% of the other and still be obtained accurately in the analysis. However, at amplitude ratios of 20:1, successful analysis to better than 50% accuracy in the smaller value has not been achieved in all trials.

In a number of instances we have analyzed for three components, using noisy two-component data. If the number of counts is sufficiently high, it has always been possible to recognize that the third component is spurious. A common finding is that, if the number of counts is sufficiently high, the third component shows a large

TABLE II

DATA SYNTHESIZED FROM $f = 1.000e^{-t/12.0} + 1.000e^{-t/18.0}$

Total counts in F	Maximum value of F in counts	Parameters found			
		α_1	τ_1	α_2	τ_2
1.60×10^6	31.1×10^3	0.78	18.8	1.21	12.6
3.19×10^6	62.1×10^3	1.10	17.7	0.89	11.5
4.79×10^6	93.2×10^3	0.90	18.3	1.10	12.3
6.39×10^6	124×10^3	1.01	18.0	0.99	11.9

TABLE III

DATA SYNTHESIZED FROM $f = 0.050e^{-t/12} + 0.050e^{-t/18}$
15.5×10^4 COUNTS IN MAXIMUM VALUE OF F 8×10^6
TOTAL COUNTS IN F

Iteration No.	Parameters found			
	α_1	τ_1	α_2	τ_2
None	0.068	17.1	0.032	10.4
First	0.054	17.7	0.046	11.7
Second	0.048	18.0	0.051	12.1
Third	0.046	18.2	0.054	12.3
Fourth	0.045	18.2	0.054	12.4
Fifth	0.045	18.3	0.055	12.4
Sixth	0.044	18.3	0.056	12.4
Seventh	0.044	18.3	0.056	12.4
Eighth	0.044	18.3	0.056	12.4

value of τ_3 and a small value of α_3, mimicking a small base line drift. Thus, in the last case of Table II, after 18 iterations, analysis for three components gave $\alpha_1 = 1.03$, $\tau_1 = 17.9$, $\alpha_2 = 0.98$, $\tau_2 = 11.9$, $\alpha_3 = 0.0002$, $\tau_3 = 52.1$. In some instances analysis yielded a third component with small negative values of α_3 and τ_3, which can be interpreted in a similar manner.

Table III illustrates the approach to stability. It demonstrates the importance of the cut-off correction and gives an example of how the iterative procedure successively alters the analyzed parameters until a self-consistent set is obtained.

THREE COMPONENT DATA

Table IV shows a typical analysis for three components.

The values in Table IV stabilized to three or more significant figures after thirty-six iterations.

TABLE IV

DATA SYNTHESIZED FROM $f = 1.000e^{-t/8} + 1.000e^{-t/12} + 1.000e^{-t/18}$

Total counts in F	Maximum value of F	Parameters found					
		α_1	τ_1	α_2	τ_2	α_3	τ_3
2.02×10^7	4.15×10^5	0.76	18.5	0.99	13.6	1.24	8.25
3.03×10^7	6.23×10^5	0.84	18.3	0.96	13.2	1.20	8.20
3.64×10^7	7.48×10^5	0.90	18.2	0.98	12.7	1.11	8.06
5.06×10^7	1.04×10^6	0.92	18.2	0.97	12.6	1.10	8.06
6.07×10^7	1.25×10^6	0.92	18.2	0.96	12.7	1.02	8.10

RESIDUAL EQUATION

We define a set of determinants, D_i, as follows.

If the system is analyzed in terms of one component, we define

$$D_1 = G_1 = \alpha_1\tau_1 . \qquad (14)$$

If the system is analyzed in terms of two components, we define

$$D_2 = \begin{vmatrix} G_1 & G_2 \\ G_2 & G_3 \end{vmatrix} \qquad (15)$$

and, in general, if the system is analyzed for N components we define

$$D_N = \begin{vmatrix} G_1 & \cdots & G_N \\ G_2 & \cdots & G_{N+1} \\ \vdots & & \\ G_N & \cdots & G_{2N} \end{vmatrix} . \qquad (16)$$

It is shown in Appendix 2 that

$$D_1 = \alpha_1\tau_1 \tag{17 a}$$

$$D_2 = \alpha_1\alpha_2\tau_1\tau_2(\tau_1 - \tau_2)^2 \tag{17 b}$$

$$D_3 = \alpha_1\alpha_2\alpha_3\tau_1\tau_2\tau_3(\tau_1 - \tau_2)^2(\tau_1 - \tau_3)^2(\tau_2 - \tau_3)^2 \tag{17 c}$$

and, in general,

$$D_n = \prod_{i=1}^{n} \alpha_i\tau_i \prod_{j>k}^{n} (\tau_k - \tau_j)^2 \tag{18}$$

$$D_{N+1}/D_N = \alpha_{N+1}\tau_{N+1} \prod_{i=1}^{N} (\tau_{N+1} - \tau_i)^2 \tag{19}$$

and

$$\alpha_{N+1} = \frac{1}{\tau_{N+1} \prod_{i=1}^{N} (\tau_{N+1} - \tau_i)^2} \frac{D_{N+1}}{D_N}. \tag{20}$$

If a set of experimental data has been analyzed in terms of N components, equation 20 provides a reinterpretation of the theorem of Appendix 1. For ideal, errorless data, $D_{N+1} = 0$ and therefore $\alpha_{N+1} = 0$. However, because of experimental error a computed value of D_{N+1} will not vanish.

Even though equation 20 is based on a noise-free system of $N + 1$ components, the equation is still useful in considering the probability that a noisy system, that has already been analyzed in terms of N components, may in fact have an additional decay. It should be emphasized, however, that the procedure to be described, by itself, does not prove or disprove the existence of an additional component, but is simply a guide in the analysis.

It is clear, on physical grounds, that, given an analysis in terms of N decays, the amplitude of a presumed $N + 1$ component will depend on the assumed τ_{N+1}. In fact the procedure becomes meaningless if τ_{N+1} is too close to one of the values $\tau_1, \tau_2 \cdots \tau_N$, or if τ_{N+1} approaches zero.

α_{N+1}, as a function of τ_{N+1}, has minima at points between $\tau_1, \tau_2, \cdots \tau_N$. These minimal values of α_{N+1}, when compared with the values of $\alpha_1, \alpha_2, \cdots \alpha_N$, may offer a clue as to whether a significant $(N + 1)^{st}$ component exists. Our experience to date indicates that if the minimal values of α_{N+1} are small compared to $\alpha_1, \alpha_2, \cdots \alpha_N$, then an $(N + 1)^{st}$ component is lacking, while if it is comparable to one of the set $\alpha_1, \cdots \alpha_N$, a nonnegligible $(N + 1)^{st}$ component is present.

DISCUSSION

The simulated analyses show that the method of moments is promising as a means of analyzing decay curves. However, at present, its chief merit is its function as a guide in the construction of equipment. The results indicate that good signal-to-noise ratios must be used for multicomponent analyses. For a monophoton technique this requires a large number of counts, perhaps larger than that which workers have customarily obtained to date. This, in turn, requires exciting lamps that are stable and which flash at high repetition rates, plus high-speed equipment for collecting data. For other techniques other ways of achieving good signal-to-noise ratios must be used. These may involve averaging of repetitive signals.

It is not the function of this paper to discuss techniques of measurement. The reader is referred to the paper of Merkelo et al (24) for a recent promising instance of high-speed technique.

No matter what technique is used to collect the data, one must know how much is needed. The criterion should be that the results of an analysis of the data not vary as the precision is improved.

Finally, we wish to point out that the present method is applicable to any linear, causal system whose response to a delta function is the sum of exponentials. *E* and *F* are simply the input and output of the system.

The authors thank Mrs. Barbara Wulf for technical assistance.

This work was supported by Public Health Service grant CA 10872.

APPENDIX 1

Theorem: The necessary and sufficient condition for the sample to have N components is that N be the smallest number for which

$$\Delta_{N+1} \equiv \begin{vmatrix} G_1 & G_2 & \cdots & G_{N+1} \\ G_2 & G_3 & \cdots & G_{N+2} \\ \vdots & & & \\ G_{N+1} & G_{N+2} & \cdots & G_{2N+1} \end{vmatrix} = 0.$$

Proof:

Let us define the N dimensional vectors, v_s, by

$$v_1 = (\alpha_1{}^{1/2}\tau_1{}^{1/2}, \alpha_2{}^{1/2}\tau_2{}^{1/2}, \cdots, \alpha_N{}^{1/2}\tau_N{}^{1/2})$$

$$v_2 = (\alpha_1{}^{1/2}\tau_1{}^{3/2}, \alpha_2{}^{1/2}\tau_2{}^{3/2}, \cdots, \alpha_N{}^{1/2}\tau_N{}^{3/2})$$

$$\vdots$$

$$v_s = (\alpha_1{}^{1/2}\tau_1{}^{s-1/2}, \alpha_2{}^{1/2}\tau_2{}^{s-1/2}, \cdots, \alpha_N{}^{1/2}\tau_N{}^{s-1/2})$$

The vectors, $v_1, \cdots v_N$ are linearly independent while the vectors $v_1, \cdots v_N, v_{N+1}$ are linearly dependent. The necessary and sufficient condition for a set of vectors to be linearly dependent is that the Gram determinant, $| v_i \cdot v_j |$, vanish.

But

$$\begin{aligned} \nu_i \cdot \nu_j &= \sum \alpha_n {\tau_n}^{i+j-1} \\ &= G_{i+j-1} \end{aligned}$$

from which the theorem follows.

APPENDIX 2

We show that, if one component is present,

$$D_1 = \alpha_1 \tau_1$$

and if two components are present,

$$D_2 = \alpha_1 \alpha_2 \tau_1 \tau_2 (\tau_1 - \tau_2)^2$$

and if three components are present,

$$D_3 = \alpha_1 \alpha_2 \alpha_3 \tau_1 \tau_2 \tau_3 (\tau_1 - \tau_2)^2 (\tau_1 - \tau_3)^2 (\tau_2 - \tau_3)^2$$

and, in general, if n components are present,

$$D_n = \prod_{i=1}^{n} \alpha_i \tau_i \prod_{j>k}^{n} (\tau_k - \tau_j)^2.$$

Proof: If n components are present

$$D_n = \begin{vmatrix} \sum_{i=1}^{n} \alpha_i \tau_i & \sum_{i=1}^{n} \alpha_i {\tau_i}^2 & \cdots & \sum_{i=1}^{n} \alpha_i {\tau_i}^n \\ \sum_{i=1}^{n} \alpha_i {\tau_i}^2 & \sum_{i=1}^{n} \alpha_i {\tau_i}^3 & \cdots & \sum_{i=1}^{n} \alpha_i {\tau_i}^{n+1} \\ \vdots & & & \\ \sum_{i=1}^{n} \alpha_i {\tau_i}^n & \sum_{i=1}^{n} \alpha_i {\tau_i}^{n+1} & \cdots & \sum_{i=1}^{n} \alpha_i {\tau_i}^{2n-1} \end{vmatrix}$$

$$= \begin{vmatrix} \alpha_1 & \alpha_2 & \cdots & \alpha_n \\ \alpha_1 \tau_1 & \alpha_2 \tau_2 & \cdots & \alpha_n \tau_n \\ \vdots & & & \\ \alpha_1 {\tau_1}^{n-1} & \alpha_2 {\tau_2}^{n-1} & \cdots & \alpha_n {\tau_n}^{n-1} \end{vmatrix} \cdot \begin{vmatrix} \tau_1 & {\tau_1}^2 & \cdots & {\tau_1}^n \\ \tau_2 & {\tau_2}^2 & \cdots & {\tau_2}^n \\ \tau_n & {\tau_n}^2 & \cdots & {\tau_n}^n \end{vmatrix}$$

$$= (\alpha_1 \cdot \alpha_2 \cdot \ldots \alpha_n)(\tau_1 \tau_2 \ldots \tau_n) {S_n}^2$$

where

$$S_n = \begin{vmatrix} 1 & 1 & \cdots & 1 \\ \tau_1 & \tau_2 & \cdots & \tau_n \\ {\tau_1}^2 & {\tau_2}^2 & \cdots & {\tau_n}^2 \\ \vdots & & & \\ {\tau_1}^{n-1} & {\tau_2}^{n-1} & \cdots & {\tau_n}^{n-1} \end{vmatrix}.$$

S_n is the Vandermonde determinant (25) and is equal to $\prod_{j>k} (\tau_j - \tau_k)$.

$$D_n = \prod_{i=1}^{n} \alpha_i \tau_i \prod_{j>k}^{n} (\tau_k - \tau_j)^2.$$

Received for publication 25 May 1969 and in revised form 31 July 1969.

REFERENCES

1. Stryer, L. 1968. *Science (Washington)*. **162**:526.
2. Tao, T. 1969. *In* Molecular Luminescence. E. C. Lim, editor. W. A. Benjamin, Inc., New York. 851.
3. Chen, R. F. 1967. *In* Fluorescence, Theory, Instrumentation and Practice. G. G. Guilbault, editor. Marcel Dekker, Inc., New York. 443.
4. Wahl, P., and H. Lami. 1967. *Biochim. Biophys. Acta*. **133**:233.
5. Weber, G. 1969. Abstracts of the Biophysical Society 13th Annual Meeting. **9**:23.
6. Chen, R. 1968. *Arch. Biochem. Biophys.* **128**:163.
7. Birks, J. B., and D. J. Dyson. 1961. *J. Sci. Instrum.* **38**:282.
8. Bauer, R. K. 1963. *Z. Naturforsch.* **189**:718.
9. Spencer, R. D., W. M. Vaughan, and G. Weber. 1969. *In* Molecular Luminescence. E. C. Lim, editor. W. A. Benjamin, Inc., New York. 607.
10. Spencer, R. D., and G. Weber. 1969. *Ann. N. Y. Acad. Sci.* **158**:361.
11. Müller, A., R. Lumry, and M. S. Walker. 1969. *Photochem. Photobiol.* **9**:113.
12. Brody, S. S. 1957. *Rev. Sci. Instrum.* **28**:1021.
13. Amata, C. D., and P. K. Ludwig. 1967. *J. Chem. Phys.* **47**:3540.
14. Nicholson, W. J., and F. Fortoul. 1967. *Biochim. Biophys. Acta.* **143**:577.
15. Sterngraber, O. J., and I. B. Berlman. 1963. *Rev. Sci. Instrum.* **34**:524.
16. Wahl, Ph. 1969. *Biochim. Biophys. Acta.* **175**:55.
17. Koechlin, Y. 1964. *Trans. N. Y. Acad. Sci.* **27**:227.
18. Haugland, R. P., and L. Stryer. 1967. *In* Conformation of Biopolymers. G. N. Ramachandran, editor. Academic Press, Inc., New York. 321.
19. Hundley, L., T. Coburn, E. Garwin, and L. Stryer. 1967. *Rev. Sci. Instrum.* **38**:488.
20. Bay, Z. 1950. *Phys. Rev.* **77**:419.
21. Bennett, R. G. 1964. *J. Chem. Phys.* **41**:3037.
22. Parker, C. A. 1968. Photoluminescence of Solutions. Elsevier, Paris.
23. Hildebrand, F. B. 1956. Introduction to Numerical Analysis. McGraw-Hill Book Co., New York. 378.
24. Merkelo, H., S. R. Hartmann, T. Mar, G. S. Singkel, and Govendjee. 1969. *Science (Washington)*. **164**:301.
25. Mostow, G. D., J. H. Sampson, and J. P. Meyer. 1963. Fundamental Structures of Algebra. McGraw-Hill Book Co., New York. 313.