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ANALYSIS OF FLUORESCENCE ANISOTROPY DECAYS BY A LEAST SQUARE METHOD

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A method of fluorescence anisotropy decay analysis is described in this work. The transient anisotropy $r^{ex}(t)$ measured in a photocounting pulsefluorimeter is fitted by a non linear least square procedure to the ratio of convolutions of the apparatus response function $g(t)$ by sums of appropriate exponential functions. This method takes rigorously into account th apparatus response function and is applicable to any shape of the later as well as to any values of fluorescence decay times and correlation times. The performances of the method have been tested with data simulated from measured response functions corresponding to an air lamp and a high pressure nitrogen lamp. The statistical standard errors of the anisotropy decay parameters have been found to be smaller than the standard errors previously calculated for the moment method. A systematic error $\Delta\tau$ in the fluorescence decay time entailed an error $\Delta\theta$ in the correlation time such as $\Delta\theta/\theta < \Delta\tau/\tau$. By this method, good fitting of experimental data have been achieved very conveniently and accurately.

1. Introduction

According to the theory of the fluorescence depolarization of solutions, the anisotropy of the fluorescence excited by an infinitely shortlight pulse decays as a sum of exponential functions, the parameters of which depend on the characteristics of the rotational Brownian motion of the emitting chromophores. This property has been used in a number of works in which transient anisotropy has been determined in a pulse fluorimeter, and has provided informations on the size, the shape, the flexibility and the interactions of macromolecules in solution [1–4]. More recently this kind of measurements have allowed to study the microviscosity of lipid vesicles and membranes [5,6].

In a recent work [9], we studied the standard errors in the anisotropy decay parameters due to counting fluctuations in measurements performed by using a photocounting pulsefluorimeter. It has been shown that correlation times comprised between 0.1 τ and 100 τ, where τ is the fluorescence decay time, can be obtained with a statistical accuracy better than 10%. The experimental transient anisotropy differs from the "ideal" anisotropy decay because pulsefluorimeters have a finite response function. In several papers, this difference has been considered as negligible, and anisotropy decay parameters have been obtained by fitting directly the experimental transient curve by a sum of exponential functions [7,8]. This procedure which has the advantage of simplicity, requires that the apparatus response should bring about a sufficiently small pertu bation on the transient fluorescence. Whether this requirement is fulfilled depends on the experimental conditions. In fact, it will be shown in this work that the procedure may entail important systematic errors Some authors take into account the apparatus response function by deconvoluting the two experimental functions $s(t)$ and $d(t)$ defined as linear combinations of the two polarized components of the transien fluorescence. This procedure, which is correct in principle, accumulates the computing errors which affect the two separate deconvolution mentioned above. In order to achieve the accuracy limited by the statistica errors, one needs a better method of data analysis tha the two methods outlined above. In order to reach this aim, we set up a new method of anisotropy decay parameters determination: the transient anisotropy is fitted to the appropriated ratio of two sums of exponential functions.

In the present paper the method is described and checked with data simulated from experimental apparatus response functions. This allows an exact evaluation of the statistical standard errors in anisotropy parameters and of the systematic errors due to the anac-

curacy in the determination of the fluorescence decay time.

2. Principle of the method

2.1. Transient fluorescence

The determination of the fluorescence anisotropy decay requires the measurement of the principal components of the polarized transient fluorescence, $i^{\parallel}(t)$ and $i^{\perp}(t)$. These measurements are performed by using a pulsefluorimeter [3,4]. One then computes the following experimental functions (case of linearly polarized excitation)

$$s^{ex}(t) = i^{\parallel}(t) + 2\beta i^{\perp}(t), \tag{1}$$

$$d^{ex}(t) = i^{\parallel}(t) - \beta i^{\perp}(t), \tag{2}$$

where β is a corrective coefficient which must be introduced when, in consequence of the measuring procedure, the ratio $i^{\parallel}(t)/i^{\perp}(t)$ is not equal to the ratio of the corresponding fluorescence intensities. This is especially the case when the fast monophotons counting technique has been used [9–11]. β is given by the expressions:

$$\beta = \frac{I_{\perp}}{I_{\parallel}} \frac{\int i^{\parallel}(t)\,dt}{\int i^{\perp}(t)\,dt},$$

where $I_{\parallel}/I_{\perp}$ is the intensities ratio of the average polarized components of the fluorescence which can be obtained by relatively short time measurements as follows: the photon-counting rate in the pulse fluorimeter must be sufficiently low, and the two components must be measured alternatively and several times in order to eliminate the lamp intensity fluctuations [3]. $I_{\parallel}$ and $I_{\perp}$ are then the total counts obtained for these components.

Except for the measurement errors, the curves $s^{ex}(t)$ and $d^{ex}(t)$ are considered as being reproducible by convolution products of the type:

$$s^{c}(t) = g(t) * S(t), \tag{3}$$

$$d^{c}(t) = g(t) * D(t), \tag{4}$$

where $S(t)$ and $D(t)$ are fluorescence decays corresponding to an infinitely short excitation and $g(t)$ a function describing the temporal characteristics of the apparatus. $g(t)$ can be experimentally determined by measuring the transient fluorescence of a reference compound having a known decay time τ_R [12]. $S(t)$ is the whole fluorescence decay which is usually a sum of exponential functions, written as follows:

$$S(t) = \sum_{i=1}^{m} A_i \exp(-t/\tau_i), \tag{5}$$

where the τ_i's are the fluorescence decay constants. The values of the parameters A_i and τ_i can be determined from $s^{ex}(t)$ and $g(t)$ by one of the deconvolution methods described in the literature [13–15].

The expression of $D(t)$ depends on the nature of the emitting chromophore heterogeneity characterizing the studied sample. For simplification we will only consider here the case where all chromophores have either the same rotational brownian motion or the same fluorescence decay. Under these conditions, one can write:

$$D(t) = S(t) \cdot R(t), \tag{6}$$

where $R(t)$ is the anisotropy decay which can be written:

$$R(t) = \sum_{j=1}^{p} \alpha_j \exp(-t/\theta_j), \tag{7}$$

where θ_j are the brownian correlation times. The fundamental anisotropy is given by the expression:

$$r_0 = \sum_{j=1}^{p} \alpha_j.$$

According to expressions (5), (7), (6) may be written:

$$D(t) = \sum_{i=1}^{m} \sum_{j=1}^{p} A_i \alpha_j \exp[-t(\lambda_i + \mu_j)] \tag{8}$$

where

$$\lambda_i = 1/\tau_i, \qquad \mu_j = 1/\theta_j. \tag{9}$$

The method exposed here can be extended to the more general case where there exist in the solution studied, several kinds of fluorescent chromophores, each having its own fluorescence and anisotropy decay. In this case, which will not be studied in this work, the second member of (6) must be replaced by a sum of products, comprising as many terms as there are kinds of chromophores [2–4]. Practically, the curves $g(t)$, $i^{\parallel}(t)$, $i^{\perp}(t)$ are measured by the technique of sampling of the photoelectron arrival times [3,16,

17]. These curves are registered as histograms in identical subgrouping of a multichannel analyzer. The channels of address k of these subgroupings contain the counts $i_k^{\parallel}$, $i_k^{\perp}$, g^k respectively, which represent the number of photoelectrons detected in a time interval defined by the $(k-\frac{1}{2})h$ and $(k+\frac{1}{2})h$ times.

One then calculates the following quantities:

$$s_k^{ex} = i_k^{\parallel} + 2\beta i_k^{\perp}, \qquad d_k^{ex} = i_k^{\parallel} - \beta i_k^{\perp}, \tag{10}$$

where β has been experimentally determined.

The numbers $i_k^{\parallel}$ and $i_k^{\perp}$ are admitedly samples of random independant variables having a Poisson distribution [18]. One can then write:

$$\widehat{\text{var}}\,[i_k^{\parallel}] = i_k^{\parallel}, \qquad \widehat{\text{var}}\,[i_k^{\perp}] = i_k^{\perp}, \tag{11}$$

where $\widehat{\text{var}}\,[x]$ designates an variance estimate of a random variable x.

Let us point out that s_k^{ex} and d_k^{ex} are not poissonian random variables. By applying the law of error propagation [19] to expression (10) one obtains ‡.

$$\widehat{\text{var}}\,[s_k^{ex}] = i_k^{\parallel} + 4\beta^2 i_k^{\perp}$$

$$= (s_k^{ex}/3)\,[1+4\beta] + (2d^{ex}/3)\,[1-2\beta], \tag{12a}$$

$$\widehat{\text{var}}\,[d_k^{ex}] = i_k^{\parallel} + \beta^2 i_k^{\perp}$$

$$= (s_k^{ex}/3)\,[1+\beta] + (d^{ex}/3)\,[2-\beta], \tag{12b}$$

$$\text{cov}\,[s_k^{ex} \cdot d_k^{ex}] = i_k^{\parallel} - 2\beta^2 i_k^{\perp}$$

$$= (s^{ex}/3)\,[1-2\beta] + (2d^{ex}/2)\,[1+\beta]. \tag{12c}$$

To the experimental quantities defined by expression (10), one associates the computed expressions:

$$s_k^c = \sum_i A_i \int_{(k-1/2)h}^{(k+1/2)h} g(t) * \exp(-t\lambda_i)\,dt, \tag{13}$$

$$d_k^c = \sum_i \sum_j A_i \alpha_j \int_{h(k-1/2)}^{h(k+1/2)} g(t) * \exp[-(\lambda_i + \mu_j)\,t]\,. \tag{14}$$

‡ Remark: In the eq. (29) of ref. [9], var(s_k), was erronously set equal to s_k; according to (12) one should have written (case β=1): var$[s_k] = (5s_k - 2d_k)/3$. Consequently, there are terms missing in expressions (41) and (42) of this work. One can show however that the numerical values of these expressions as well as their limiting expressions (50) were not affected by these errors.

2.2. Determination of the anisotropy decay parameters

The parameters α_j and μ_j of $R(t)$ are determined by fitting the set of calculated ratios:

$$r_k^c = d_k^c / s_k^c, \tag{15}$$

with the experimental quantities:

$$r_k^{ex} = d_k^{ex} / s_k^{ex}. \tag{16}$$

According to expressions (13) and (14), (15) may be written:

$$r_k^c = \frac{1}{s_k^c} \sum_{i=1}^{m} \sum_{j=1}^{p} A_i \alpha_j \int_{(k-1/2)h}^{(k+1/2)h} g(t) * \exp[-(\lambda_i + \mu_j)\,t]\,dt. \tag{17}$$

In this expression, s_k^c is known since the A_i's and τ_i's are known.

According to the least square principle [19], one has to find the α_j's and μ_j's which minimize the sum:

$$\chi_2 = \sum_{k=1}^{n} (r_k^c - r_k^{ex})^2 / \hat{V}_k, \tag{18}$$

where n is the number of experimental points in each of the experimental curves, and $\hat{V}_k$ a variance estimate of the random variable represented by its sample r_k^{ex}.

Taking into account (10) and (11), the law of error propagation [19] applied to (16) leads to:

$$\hat{V}_k = \frac{1}{3 s_k^{ex}} [1 + \beta + 3\beta r_k^{ex} - 3(r_k^{ex})^2 - 2(2\beta - 1)(r_k^{ex})^3], \tag{19}$$

which reduces to a previously obtained expression in the case $\beta = 1$ [9].

The parameters α_j, μ_j which minimize χ_2 can be obtained by a method of non linear least square as the Marquard's method [19]. These are iterative methods which at each iteration, need the computation of the χ_2 curvature matrix A, the elements of which can be written in our case:

$$A_{uv} = \sum_{k=1}^{n} \frac{\partial s_k^c}{\partial P_u} \frac{\partial r_k^c}{\partial P_v} \frac{1}{\hat{V}_k}, \qquad \begin{matrix} 1 < u < 2p \\ 1 < v < 2p \end{matrix} \tag{20}$$

where P_u and P_v are the parameters to be determined which designate the α_j's or μ_j's.

The computer libraries usually contain non linear least squares sub-programs. The user has to provide the derivatives contained in (20). From (17) one can write:

$$\frac{\partial r_k^c}{\partial \alpha_j} = \frac{1}{s_k^c} \sum_{i=1}^{m} A_i \int_{h(k-1/2)}^{h(k+1/2)} g(t) * \exp[-(\lambda_i + \mu_j)t]\, dt, \quad (21)$$

$$\frac{\partial r_k^c}{\partial \mu_j} = -\frac{\alpha_j}{s_k^c} \sum_{i=1}^{m} A_i \int_{h(k-1/2)}^{h(k+1/2)} g(t) * t \exp[-(\lambda_i + \mu_j)t]\, dt. \quad (22)$$

Algorithms for the computation of expressions (21) and (22) are given in appendix 1 and 2.

2.3. Statistical errors on the anisotropy decay parameters

A variance estimate of P_u is given by the diagonal element of the error matrix defined by: $\epsilon = A^{-1}$ which can be written [19]:

$$\mathrm{var}[P_u] = \epsilon_{uu}. \quad (23)$$

The statistical errors on α_j and θ_j are then given by:

$$\Delta\alpha_j = \sqrt{\mathrm{var}(\alpha_j)}, \qquad \Delta\theta_j/\theta_j = \sqrt{\mathrm{var}(\mu_j)/\mu_j}. \quad (24)$$

In the case where the fluorescence decays and the anisotropy decays are single exponential functions, these errors can be compared to the corresponding error computed for the moment method, the analytical expression of which have been given in a recent work [9]. According to equations (43) and (49) of this work, one can write:

$$\Delta\theta/\theta = A(r_0, \theta/\tau) N^{-1/2} \times [1 + B(r_0, \theta/\tau, N/N')\, \sigma(g)^2/\tau^2]^{1/2}, \quad (25)$$

$$\Delta r_0 = C(r_0, \theta/\tau) N^{-1/2} \times [1 + D(r_0, \theta/\tau, N/N')\, \sigma(g)^2/\tau^2]^{1/2}, \quad (26)$$

where A, B, C, D are given by expressions (41), (42) and (48) of the same work, r_0 is the anisotropy decay at zero time (in this case, $\alpha_1 = r_0$, $\alpha_i = 0$ for $i \neq 1$) N and N', the total counts in $s(t)$ and $g(t)$ respectively, $\sigma(g)$ the standard deviation of the distribution corresponding to $g(t)$. When $\sigma(g)/\tau$ is sufficiently small, (25) and (26) can be reduced to

$$\Delta\theta/\theta = A(r_0, \theta/\tau) N^{-1/2}, \quad (27)$$

$$Ar_0 = C(r_0, \theta/\tau) N^{-1/2}. \quad (28)$$

These errors are then independent from the shape of the $g(t)$ function

2.4. The computer program

A flow diagram of the program is given on fig. 1. The reference transient fluorescence f_k ($k = 1,n$), its decay time τ_R, the time interval h, the components $i_k^{\parallel}$, $i_k^{\perp}$ of the fluorescence to be analysed, and the correct intensity ratio $I_{\parallel}/I_{\perp}$ are read by the program.

The program computes β, s_k^{ex}, d_k^{ex}, r_k^{ex}, and $\hat{V}_k$. The parameter values of the A_i's and τ_i's which characterize $S(t)$, are then entered with initial "guessed" values α_j^0, θ_j^0 of $R(t)$. The program calls the non linear least square subroutine which determines the $\hat{\alpha}_j$'s and $\hat{\theta}_j$'s estimated by successive iterations. We used here a subroutine based on a modified Marquardt method, which belonged to the Harwell library [20].

The final estimate values of the parameters are writ-

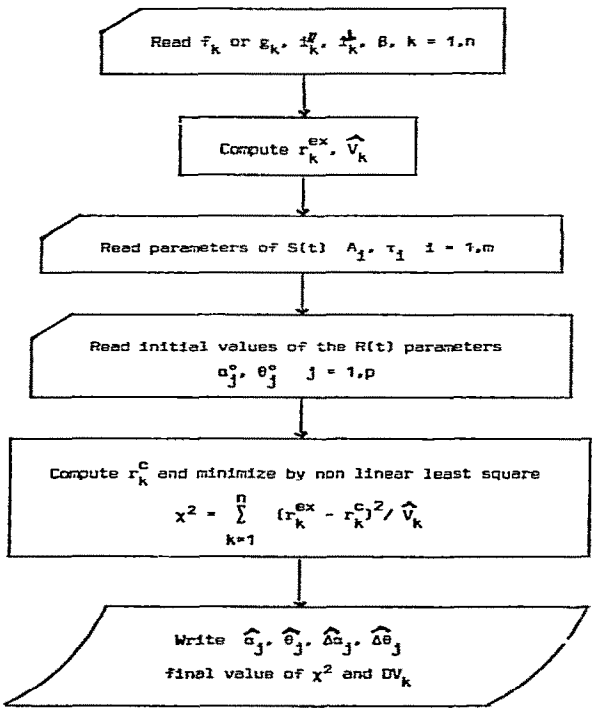

Fig. 1. Flow chart of the least square program used for anisotropy decay analysis.

Table 1
Response function characteristics, obtained by measureing the fluorescence transient of 1,1,4,4 tetraphenyl butadiene ($\tau_R = 1.76$ ns), and applying the algorithm given in ref. [12]. h = time separation between two analyser channels. λ_{ex} = excitation wavelength defined by an interference filter. λ_{em} = emission wavelength defined by a high pass filter. N' = total count in $g(t)$. T_R = Rise time between 10% and 90% of the $g(t)$ maximum. $L_{1/2}$, $L_{1/10}$, $L_{1/100}$ widths of $g(t)$ at 1/2, 1/10, 1/100 of the intensity maximum. $\sigma(g)$ = standard deviation of the distribution corresponding to $g(t)$

Lamp	h ns/cl	λ_{ex} (nm)	λ_{em} (nm)	N' $\times 10^{-5}$	T_R (ns)	$L_{H/2}$ (ns)	$L_{H/10}$ (ns)	$L_{H/100}$ (ns)	$\sigma(g)$ (ns)
Air no 1	0.238	336	> 438	9.30	1.24	1.43	3.33	7.14	2.65
Air no 2	0.588	336	> 438	9.83	1.47	1.76	4.116	7.88	4.17
Nitrogen	0.476	367	> 526	5.67	1.05	1.43	4.76	17.14	8.8

ten with their statistical errors, the final χ^2 value, and the deviation function defined by

$$DV_k = (r_k^{ex} - r_k^c)/\sqrt{\tilde{V}_k}, \qquad k = 1, n. \tag{29}$$

The parameter values are satisfactory if χ_2 is close to one and if DV_k oscillates around 0 with an average amplitude equal to one.

3. Testing of the method with synthetic data

3.1. Data synthesis

We first checked the proposed method, with synthetic data obtained in the following way. The transient fluorescence $f(t)$ of a sample of tetraphenyl 1-1-4-4-butadiene (TPB) having a fluorescence decay time of 1.76 ns [21] has been measured with the pulse fluorimeter previously described [12]. The exciting lamps were alternatively a spark bursting into air at atmosheric pressure and a spark bursting into nitrogen at a pressure of 15 kg per cm^2. The detecting photomultiplier was a RCA 8850. The conditions of measurements and the characteristics of the apparatus function $g(t)$ obtained as previously described [12], are given on table 1. These functions are represented on figs. 2 and 3.

3.2. Data simulation

We checked the proposed method with synthetic data obtained in the following way: the product of convolution $s^T(t)$ and $d^T(t)$ of the exponential functions $S(t)$ and $S(t) \cdot R(t)$, by the apparatus response $g(t)$, were computed by using the algorithm expressed

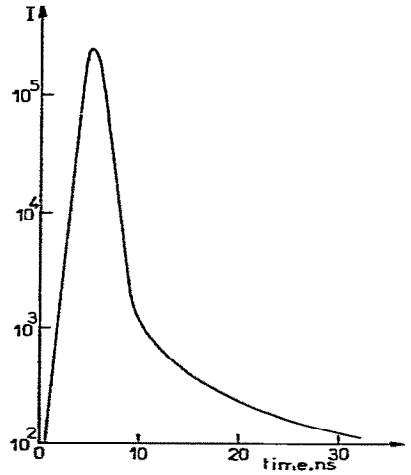

Fig. 2. Response function $g(t)$ of the photon counting pulse-fluorimeter obtained with the air lamp, record no 1.

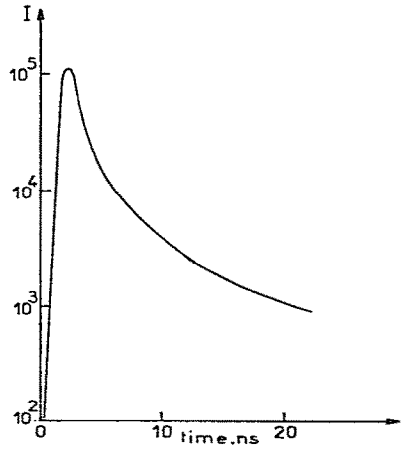

Fig. 3. Response function $g(t)$ of the photon counting pulse-fluorimeter obtained with a nitrogen lamp.

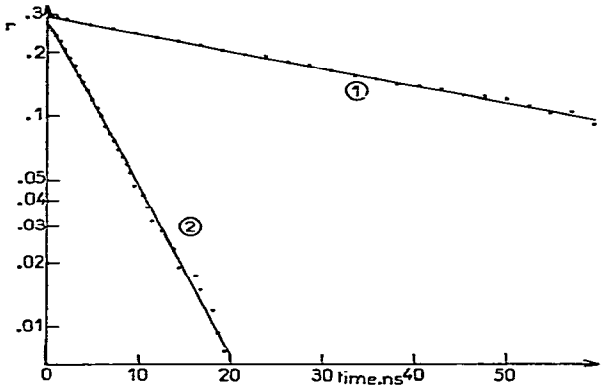

Fig. 4. Transient anisotropy simulated without (continuous line) and with (points) fluctuations. The air lamp no 1 was used; the fluorescence decay time was $\tau = 20$ ns and the correlation time were ① $\theta = 50$ ns and ② $\theta = 5$ ns.

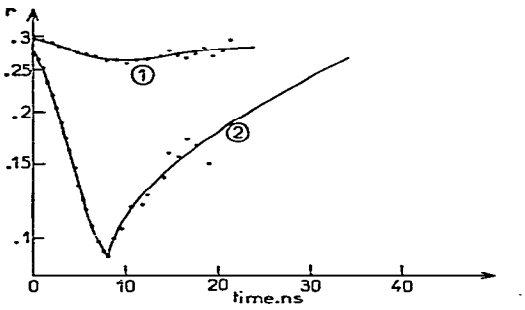

Fig. 5. Same as fig. 4 except that $\tau = 2$ ns.

in formula (A.21). To the counts s_k^T and d_k^T, one could add respectively the random numbers V_k^s and V_k^d defined as follows:

$$V_k^s = V_k^{\parallel} + 2V_k^{\perp}, \qquad V_k^d = V_k^{\parallel} - V_k^{\perp}, \tag{30}$$

where $V_k^{\parallel}$ and $V_k^{\perp}$ were random numbers which simulated the poissonian fluctuations of $i_k^{\parallel}$ and $i_k^{\perp}$. According to (10) (case $\beta = 1$) one had:

$$i_k^{\parallel} = \tfrac{1}{3}(s_k^T + 2d_k^T), \qquad i_k^{\perp} = \tfrac{1}{3}(s_k^T - d_k^T). \tag{31}$$

As the $i_k^{\parallel}$ and $i_k^{\perp}$ values were high (greater than 100), $V_k^{\parallel}$ and $V_k^{\perp}$ could be generated with pseudo random numbers of gaussian distribution having variances equal to $i_k^{\parallel}$ and $i_k^{\perp}$ respectively.

The function $r^T(t) = d^T(t)/s^T(t)$ was then analysed according to the method described above.

In order to compare the method proposed here with the method which consists in analysing $r^{ex}(t)$ as a sum of exponentials, another program was set up which minimized the following expression:

$$\chi^2 = \frac{1}{n}\sum_{n=1}^{n}(r_k^T - R_k)^2/V_k.$$

In this last program, we used the same non linear least square subprogram.

3.3. Results obtained from monoexponential anisotropy decays

The fluorescence was also assumed to be monoexponential in these simulations; τ was alternatively chosen equal to 20 ns and 2 ns. The total count N in $s(t)$ was fixed to 2×10^7. The fundamental anisotropy r_0 was equal to 0.3.

3.3.1. Shape of the anisotropy transient $r^T(t)$

The figs. 4, 5 and 6 show the variation of $r^T(t)$ in semi logarithmic coordinates. The continuous line and the points represent the result of the simulation without and with added fluctuations respectively.

The excitation sources were the air lamp (record no 1) in the data of figs. 4 and 5 and the nitrogen lamp for fig. 6. The fluorescence decay time was 20 ns for figs. 4 and 6, and 2 ns for fig. 5. It can be seen on figs. 4, 5 and 6 that the semi logarithmic representation of

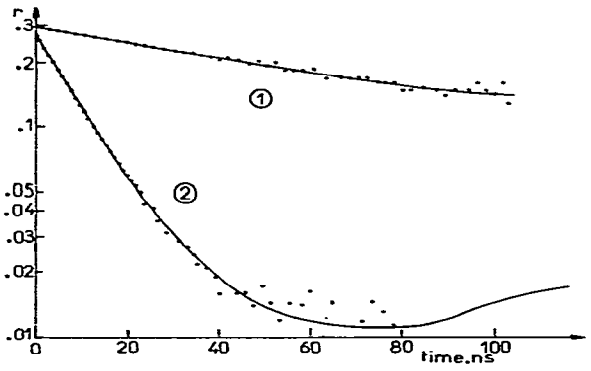

Fig. 6. Same as fig. 4 except that excitation source was the nitrogen lamp and the correlation times were ① $\theta = 100$ ns, ② $\theta = 10$ ns.

Table 2
Errors due to directly decomposing the experimental transient anisotropy curve into an exponential function. Comparison with the method proposed here. The fluctuations were added to the simulated curves. τ, r_0, θ are the parameters used in the simulation, ΔT the time range of analysis commencing to the maximum of the reference transient maximum. $\hat{r}_0$, $\hat{\theta}$ the estimates resulting from analyses, Δr_0 and $\Delta\theta$ the statistical errors

Simulations				Direct analysis of the transient anisotropy								Method of this work						
Lamp	τ (ns)	r_0	θ (ns)	ΔT (ns)	$\hat{r}_0$	$\hat{\theta}$ (ns)	χ_2	ΔT (ns)	$\hat{r}_0$	$\hat{\theta}$ (ns)	χ_2	ΔT (ns)	$\hat{r}_0$	$\hat{\theta}$ (ns)	χ_2	Δr_0	$\Delta\theta$ (ns)	
Air no 1	2	0.3	5	64	0.291	7.5	39.5	7.1	0.267	6.2	3.3	64	0.3003	4.98	0.86	0.001	0.03	
Air no 1	2	0.3	50	64	0.294	138	4.56	7.1	0.297	58.3	0.68	64	0.3002	48.8	0.85	0.0004	1.3	
Air no 1	20	0.3	5	64	0.305	5.49	1.55	11.9	0.305	5.5	4.1	64	0.3002	4.98	0.87	0.001	0.03	
Air no 1	20	0.3	50	64	0.301	51.9	0.86	11.9	0.301	51.5	0.76	64	0.2996	50.3	0.86	0.0004	0.22	
Nitrogen	20	0.3	10	127	0.282	13.3	3.36	19	0.276	12.7	1.13	127	0.3002	9.97	0.86	0.0008	0.05	
Nitrogen	20	0.3	100	127	0.298	123	1.34	19	0.297	120	0.82	127	0.2995	100.8	0.86	0.0004	0.6	

$r^T(t)$ is not generally a straight line, which must be attributed to the influence of the non infinitely short temporal distribution of the response $g(t)$.

The comparison of figs. 4 and 6 shows that the nitrogen lamp gives a more pronounced deviation from linearity than the air lamp. This comes from the more dissymmetrical shape and the longer trail of $g(t)$ when nitrogen flash is used. Comparison of fig. 4 and fig. 5 shows that the value of the fluorescence decay time strongly determines the range of the anisotropy curve which is significant. In the case of $\tau = 2$ ns for example, one sees that the systematic deformation due to the flash tail and the amplitude of random fluctuations hinder all significant analysis for $t > 10$ ns. This figure agrees with the prediction made in our preceding work, where it was established that for $\theta > \tau$, the optimum range of decay analysis by the slope method was $\Delta t \approx 5\tau$ (expression (86) of ref. [9]).

3.3.2. Comparison between the method proposed and the direct fitting of the experimental transient anisotropy with exponential functions

The synthetic anisotropy functions represented on figs. 4, 5 and 6 were fitted with exponential functions according to the method used by a number of authors [7,8]. The results obtained with the data synthesized with added random fluctuations are brought on table 2.

By comparing the θ and r_0 values introduced in the data synthesis, with the estimate values θ and r_0 obtained by the least square analysis, one can evaluate the systematic error due to this process. We must notice that, in the results presented in this work, the origin of the time range in which anisotropy analysis was performed, was taken at the channel corresponding to the maximum of the reference transient fluorescence. The values of χ_2 reported here include systematic difference between the shape of the transient curve $r^T(t)$ and the nearer exponential function. In order to minimize the systematic error in the parameter, the analysis has been restricted to the range of significant data, as deduced from examination of the figs. 4, 5 and 6. It can be seen from table 2 that for $\tau = 20$ ns, the systematic error is smaller with excitation by the air lamp than with the nitrogen lamp, and that for the air lamp, the error is greater with $\tau = 2$ ns than with $\tau = 20$ ns.

On the other hand, practically no systematic error was found when using the method proposed in this work to analyse the same data (see table 2).

3.3.3. Statistical errors on correlation times

This study was performed by using the analysis proposed here. As explained above, the errors $\Delta\theta/\theta$ and Δr_0 were obtained from the error matrix computed at the last iteration of the least square analysis of $r^T(t)$. In order to obtain an exact value of these errors, the $r^T(t)$ curves were synthesized without fluctuations. The excitation function correspondent to the air flash no 2 and to the nitrogen flash were used. Time ranges corresponding to 268 channels (157 ns and 127 ns) respectively) were taken for the analysis. Fig. 7 represents $\Delta\theta/\theta$ as a function of θ in the case of a 20 ns fluorescence decay time. For the two excitation functions, $\Delta\theta/\theta$ goes through a minimum for a θ value close to the fluorescence decay time. The $\Delta\theta/\theta$ values

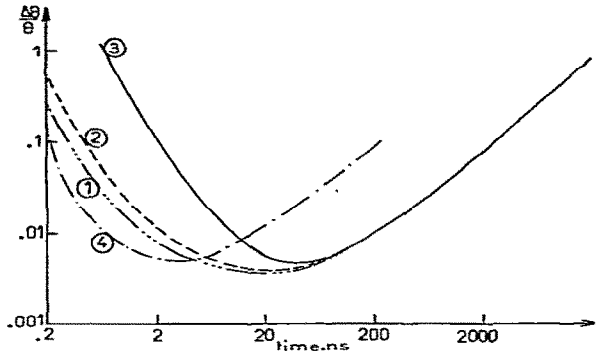

Fig. 7. The relative statistical error of the correlation time as a function of the correlation time in the case of the least square analysis. $r_0 = 0.3$. Fluorescence decay time was $\tau = 20$ ns. Excitation corresponded to ① air lamp no 2, ② nitrogen lamp, ③ analysis by the moment method, ④ air lamp no 2 and $\tau = 2$ ns for comparison. Total count in $s(t)$ was 2×10^7.

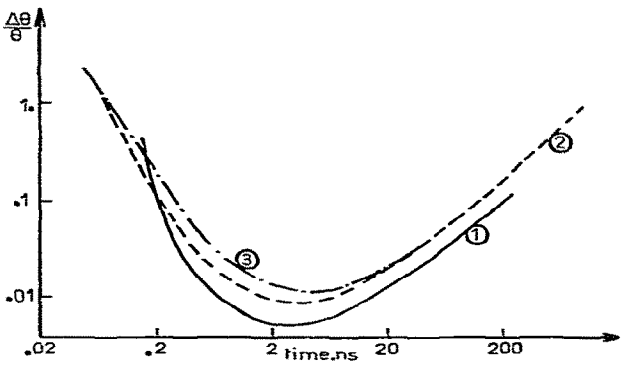

Fig. 8. Relative error of the correlation time as a function of the correlation time in the case of the analysis by the least square method. $r_0 = 0.3$. Fluorescence decay time was $\tau = 2$ ns. ① air lamp no 2, ② nitrogen lamp, ③ analysis by the moment method corresponding to excitation by the air lamp no 2.

are practically identical for both excitations when $\theta > \tau$. In the range $\theta > \tau$, $\Delta\theta/\theta$ is smaller for the air lamp than for the nitrogen lamp. On the same figure, the curve representing $\Delta\theta/\theta$ (θ) in the case of the moment method is shown. This curve was calculated from formula (27), since according to table 1, one has $\sigma^2(g)/\tau^2 \approx 0.04$ (case of the air lamp), which is a negligible value.

Fig. 7 shows that the moment method has the same accuracy as the least square method in the range $\theta > \tau$ whereas the latter is markedly better for $\theta < \tau$. The case of $\tau = 2$ ns is shown on fig. 8. Here also the minimum of $\Delta\theta/\theta$ was obtained for $\theta \approx \tau$. But for this short decay time the air lamp leads to a smaller error than the nitrogen lamp, all over the θ range. For the case of the moment method we use formula (25) in which we introduce the standard deviation $\sigma^2(g)$ of the air lamp given in table 1. Fig. 8 shows that the moment method gives a greater error on the correlation time than the least square method all over the θ range values.

The curves representing $\Delta\theta/\theta$ (θ), in the case of the air lamp and with $\tau = 2$ ns, have also been brought on fig. 7 in order to show that short θ values are ob-

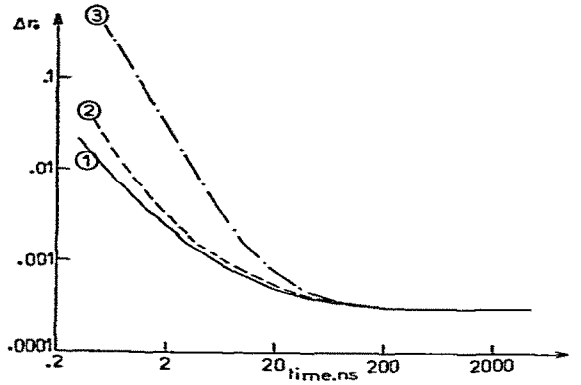

Fig. 9. Statistical error of the fundamental anisotropy in the case of the least square method $r_0 = 0.3$, $\tau = 20$ ns ① air lamp no 2, ② nitrogen lamp, ③ moment method.

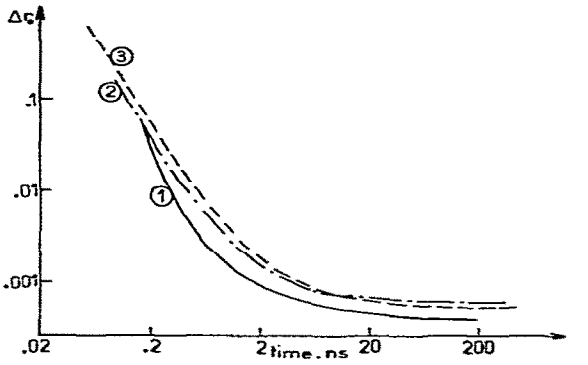

Fig. 10. Statistical error of the fundamental anisotropy in the case of the least square analysis, $r_0 = 0.3$, $\tau = 2$ ns. ① air lamp no 2. ② nitrogen lamp, ③ moment method with the air lamp excitation.

Table 3
Error of the anisotropy parameters resulting from an error in the fluorescence decay time. Transient anisotropy curves were synthesized without fluctuations, for various θ values. $r_0 = 0.3$, $\tau = 20$ ns. Analysis was performed by introducing wrong τ values of 18 ns ($\Delta\tau/\tau = -10\%$) and 22 ns ($\Delta\tau/\tau$) = +10%. Δr_0 and $(\Delta\theta/\theta)_1$ are the resulting errors. For comparison the statistical error $(\Delta\theta/\theta)_2$, computed with the correct τ value, is also reported

$\Delta\tau/\tau$	θ (ns)	Δr_0 $\times 10^3$	$(\Delta\theta/\theta)_1$ $\times 10^3$	$(\Delta\theta/\theta)_2$ $\times 10^3$	χ_2	θ (ns)	Δr_0 $\times 10^3$	$(\Delta\theta/\theta)_1$ $\times 10^3$	$(\Delta\theta/\theta)_2$ $\times 10^3$	χ_2	θ (ns)	Δr_0	$(\Delta\theta/\theta)_1$ $\times 10^3$	$(\Delta\theta/\theta)_2$ $\times 10^3$	χ_2
Air lamp no 2															
−0.1	5	−0.10	−2	5.2	0.01	20	+0.2	−3.5	3.6	0.08	100	+0.04	−9.2	5.7	0.08
+0.1		+0.1	+2		0.00		−0.1	+2.5		0.02		−0.2	+6.1		0.02
−0.1	500	+0.1	−14	20	0.01	1000	+0.1	13	41	0.00					
+0.1		−0.1	+8		0.00		+0.1	9		0.00					
Nitrogen lamp															
−0.1	5	+1.5	−14	6	0.1	20	+1.8	−25	4	0.39	100	+1.2	−40	6	0.17
+0.1		−1	+12		0.05		+1.2	+20		0.16		−0.7	+30		0.07
−0.1	500	4	−44	22	0.01	1000	+3	−42	42	0.01					
+01		0	+32		0.00		+1	+35		0.00					

tained with a better accuracy with $\tau = 2$ ns than with $\tau = 20$ ns. On the other hand, θ/τ being given, the small τ value leads to a higher $\Delta\theta/\theta$ than the large one.

3.3.4. Statistical error on r_0

Curves representing Δr_0 as functions of θ are shown on figs. 9 and 10, in the cases of $\tau = 20$ ns and 2 ns respectively.

For $\tau = 20$ ns and $\theta > 200$ ns (see fig. 9), Δr_0 has a value independent of θ ($\approx 2 \times 10^{-4}$), value which is the same for the least square method and the moment method (expression 28). In addition, both excitations lead to the same values of Δr_0. The error increased monotonously when θ decreases. In the case of the least square method, the rate of increase is higher with the nitrogen lamp than with the air lamp. The moment method leads to still higher errors.

The case of 2 ns is represented in fig. 10. The least square analysis leads to higher error with the nitrogen lamp, all over the range of θ values. The moment method error computed by using expression (26) with the standard deviation of the air lamp, is higher than the least square values obtained with the same lamp, as it is also shown on fig. 10.

Comparison of fig. 10 with fig. 9 shows that, when θ is small, the accuracy of r_0 is better with $\tau = 2$ ns than with $\tau = 20$ ns. But θ/τ given, Δr_0 is higher, in the case of $\tau = 2$ ns than in the case of 20 ns.

3.3.5. Error in correlation times resulting from an error in fluorescence decay time

In order to investigate how an error in the fluorescence decay time τ affected the error in correlation times, the $r^T(t)$ curves, synthesized with a given value of τ were compared with the fitting function $r^c(t)$ curves calculated with a value τ' different from τ.

The result of these calculations with $\tau = 20$ ns, is given in table 3. The values of χ_2, although they have no statistical meaning here, are also brought in this table, because they give a measure of the difference between the $r^T(t)$ curves and the best $r^c(t)$ curves synthesized with a wrong fluorescence decay time.

In the case of the air lamp, table 3 shows that, for θ varying between 5 ns and 10^3 ns, a 10% error in τ entails an error in θ smaller than the statistical error, and with a maximum value of 1.4%. In the case of the nitrogen lamp the same error in τ entails an error in θ which is greater than the statistical error, but remains below 4.4%.

3.4. Anisotropy decay which is a sum of two exponential functions

In this study, the anisotropy decay $R(t)$ was assumed to be a sum of two exponential functions. The excitation function was that of the nitrogen lamp; the fluorescence decay time τ was equal to 20 ns and the time range of analysis was 157 ns. The results of this

Table 4
Example of statistical errors computed with simulated anisotropy decays which were sums of two exponential functions. Fluorescence decay was a single exponential function with $\tau = 20$ ns, $\Delta\alpha_i$ and $\Delta\theta_i/\theta_i$ are statistical errors

α_1	θ_1 (ns)	α_2	θ_2 (ns)	$\Delta\alpha_1$	$\Delta\theta_1/\theta_1$	$\Delta\alpha_2$	$\Delta\theta_2/\theta_2$
0.1	30	0.2	500	0.01	0.09	0.01	0.26
0.1	20	0.2	40	0.08	0.28	0.08	0.15
0.05	6	0.25	200	0.001	0.06	0.001	0.02

study is given on table 4, where one can see the anisotropy decay parameters introduced in the simulated curves $r^T(t)$, the estimate of these parameters obtained from the least square analysis applied to transient decays with added fluctuations, and the statistical errors computed on transient anisotropy simulated without fluctuations.

The standard errors in correlation times were larger than the standard errors calculated with the same correlation time values in the case of mono-exponential decays. These errors are the larger as the two correlation time values of the decay are the closer to each other.

A similar conclusion came out from the studies of decay time standard errors [22,23].

4. Application to experimental data

Fig. 11 shows the result of an actual experiment. The sample was a solution of G actin labelled at residue cystein 373 with the Hudson-Weber label N-iodoacetyl N' (5-sulfo-1-naphtyl) ethylene diamine as explained in refs. [24] and [25]. The protein concentration was 0.3 mg/ml and the solvent composition was 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, 2 mM tris-

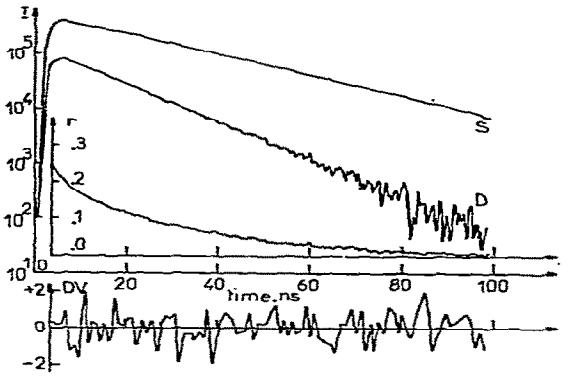

Fig. 11. Experimental transient fluorescence in polarized light of G actin labelled with 1,5 IAEDANS, temperature was 21°C. Excitation by the nitrogen lamp, ① $s(t)$, ② $d(t)$, ③$r^{ex}(t)$, ④ deviation function of $r^{ex}(t)$.

HCl pH 8. Temperature was 21°C. Excitation was provided by the nitrogen lamp, the apparatus response function of which is given in table 1.

The analysis of the transient fluorescence $s(t)$ was performed by the modulation function method [26]. The parameters of the anisotropy decay were obtained by the method described in this work. These parameters, their statistical errors computed from the error matrix elements as well as the χ_2 value are gathered in table 5. The deviation function relative to $r^{ex}(t)$ has also been drawn on fig. 11.

5. Conclusion

The method of fluorescence anisotropy decay analysis described in this work takes into account the apparatus response function. It can be used with any apparatus response function and in a wide range of fluorescence decay time and correlation time values.

Table 5
Analysis by the least square method proposed in this work of the anisotropy decay of a labelled G actin aqueous solution. The label was 1.5 IAEDANS, temperature 21°C; actine concentration 0.3 mg/ml; solvent composition: 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, 2 mM Tris-HCl, pH 8.0. ΔT: time range of the analysis, N: total count in the $s(t)$ curve; $\bar{r}$ is the average anisotropy. θ_i, α_i, $\Delta\theta_i$ and $\Delta\alpha_i$ are estimates obtained by the least square method

	ΔT (ns)	N $\times 10^{-6}$	τ (ns)	α_1	θ_1 (ns)	α_2	θ_2 (ns)	r_0	$\bar{r}$	$\Delta\alpha_1$	$\Delta\theta_1$ (ns)	$\Delta\alpha_2$	$\Delta\theta_2$ (ns)	χ_2
Actine G 0.3 mg/ml	130	22.5	20.8	0.068	1.85	0.219	27.4	0.287	0.133	0.003	0.14	0.001	0.22	1.1

These features are not achieved in the first method mentioned in the introduction, in which the experimental transient anisotropy is fitted with a sum of exponential functions.

In our study of this last method, which we will call here the "direct method", the kth term of the square sum χ_2 was weighted by a factor proportional to the corresponding count s_k of the transient fluorescence. Since s_k decayed exponentially with k, the parameter estimates were relatively independent of the strong perturbations which affected the trail shape of the transient curve.

In the work reported in the literature, the terms of the square sum were uniformly weighted. When we performed the analysis of simulated data using the "direct method" under such a uniform weighting, we found that the values of the systematic error in the correlation times and its dependence in the analysis time range were increased, sometimes considerably.

From these studies we conclude that the direct method should be used with caution since it often leads to systematic errors which depend on the shape of the apparatus response function and consequently may vary from an experiment to another.

An interesting property of the method proposed in this work is that a systematic error in the fluorescence decay time leads to a smaller error in the correlation time. This result must be attributed to the form of the fitting function $r^c(t)$ which is a ratio of two sums of exponentials containing both the parameter values of $S(t)$. Therefore there is a compensation when the $S(t)$ parameters change, which reduces the change of $r^c(t)$. This compensation is all the more effective as the narrower and the more symmetrical the response function $g(t)$ is. If $g(t)$ tends to become infinitely short, $r^c(t)$ tends to $R(t)$ and is then independent of $S(t)$.

On the contrary, one does not expect such a compensation to occur when the anisotropy parameters are obtained by two separate deconvolutions, according to the second method which has been mentioned in the introduction of this work. In this case, the inaccuracies resulting from deconvoluting $s(t)$ and $d(t)$ add up together.

In a previous work, the standard errors $\Delta\theta/\theta$ and Δr_0 in the parameters of a monoexponential anisotropy decay, were analytically calculated from the Poisson statistics of the channel counts representing the transient fluorescence registered in a multichannel analyzer [9]. It was found that these errors were inversely proportional to the total count in the transient $s(t)$ curve, and depended on the θ/τ value and on the width of the apparatus function. $\Delta\theta/\theta$ were shown to be also approximately proportional to the inverse of the anisotropy r_0.

This study concerned the moment method and the slope method. It was found that both methods gave similar $\Delta\theta/\theta$ values when τ was high relatively to the standard deviation of the apparatus response function and θ higher than τ. Under these conditions, the method described here also leads to $\Delta\theta/\theta$ values similar to those found by the two previously studied methods.

On the other hand, when τ is smaller than the standard deviation of the response function or for whichever value of τ when θ is smaller than τ, our least square method gives smaller standard errors than the moment method. Under these conditions the slope method which for monoexponential anisotropy decays is equivalent to the "direct method", is inapplicable because it entails important systematic errors.

The fitting method described in this work has been already used satisfactorily in several of our experimental works [24,25,26]. Its accuracy and its conveniency are probably better than they are with other described methods. It will contribute to increase the usefulness of the anisotropy decay measurements in the study of biological systems.

Appendix 1

Use of a reference compound in order to determine the convolution of an exponential decay

In a previous work [12], it has been shown that $g(t)$ was not directly measurable, but could be obtained from the transient fluorescence $f(t)$ of a reference compound having a monoexponential decay of the known decay time τ_R. We show here that the determination of $g(t)$ is not necessary to compute the convolution of an exponential function or of a sum of exponential functions, which can be performed by using directly the transient fluorescence $f(t)$. A convolution product which is designated symbolically by the following expression:

$$s(t) = g(t) * \exp(-\lambda t) \qquad \text{(A.1)}$$

must be explicitely written:

$$s(t) = \int_0^t g(x) \exp[-\lambda(t-x)]\,dx. \qquad (A.2)$$

It has been shown that one can write [27]:

$$g(t) = [1/M_0(f)]\,[f(t) + \tau_R\, df(t)/dt], \qquad (A.3)$$

where $M_0(f)$ is the moment of order zero of $f(t)$.

By taking into account (A.3), (A.2) becomes:

$$s(t) = [1/M_0(f)] \qquad (A.4)$$

$$\times \left[(1-\lambda\tau_R)\int_0^t f(x)\exp[-\lambda(t-x)]\,dx + \tau_R\, f(t)\right],$$

which may be symbolically written:

$$s(t) = [1/M_0(f)]$$

$$\times\ [(1-\lambda\tau_R)\, f(t) * \exp(-\lambda t)] + \tau_R\, f(t). \qquad (A.5)$$

In the same way, we had to compute:

$$H(t) = -g(t) * t\exp(-\lambda t), \qquad (A.6)$$

which is explicitely written:

$$H(t) = -\int_0^t g(x)\,(t-x)\exp[-\lambda(t-x)]\,dx. \qquad (A.7)$$

When (A.3) is taken into account, (A.7) becomes, after a partial integration:

$$H(t) = [1/M_0(f)]$$

$$\times \left[(1-\lambda\tau_R)\int_0^t f(x)\,(t-x)\exp[-\lambda(t-x)]\,dx\right.$$

$$\left. + \tau_R \int_0^t f(x)\exp[-\lambda(t-x)]\,dx\right], \qquad (A.8)$$

which may be written symbolically:

$$H(t) = [1/M_0(f)]\,[(1-\lambda\tau_R)\, f(t) * t\exp(-\lambda t)$$

$$+ \tau_R\, f(t) * \exp(-\lambda t)]. \qquad (A.9)$$

Appendix 2

Numerical algorithm for the calculation of expression (13), (14), (21) and (22)

1) *The convolution*:

$$F(t) = \int_0^t g(x)\exp[-\lambda(t-x)]\,dx \qquad (A.10)$$

may be written

$$F(t) = F(t-h)\,e^{-\lambda h} + e^{-\lambda t}\int_{t-h}^{t} g(x)\,e^{\lambda x}\,dx. \qquad (A.11)$$

We have to compute expressions such as:

$$F_k = \int_{(k-1/2)h}^{(k+1/2)h} F(t)\,dt \qquad \text{for } k = 1, \ldots n. \qquad (A.12)$$

By replacing $F(t)$ in (A.12), by the second member of (A.11), one obtains the recurrence relation:

$$F_k = F_{k-1}\,e^{-\lambda h} + \Delta F_k, \qquad (A.13)$$

where

$$\Delta F_k = \int_{(k-1/2)h}^{(k+1/2)h} e^{-\lambda t}\left[\int_{t-h}^{t+h} g(x)\,e^{\lambda x}\,dx\right]dt. \qquad (A.14)$$

We must take into account that $g(x)$ is given by a set of channel contents of an analyser, each content being defined by:

$$G_k = \int_{(k-1/2)h}^{(k+1/2)h} g(x)\,dx, \qquad k = 1, \ldots n. \qquad (A.15)$$

From G_k and the content of neighbouring channels, one can define $g(x)$ by a local plynomial. We will limit ourself to a binomial which can be written:

$$g(x) = a_k X + b_k, \qquad (A.16)$$

where

$$X = x - (n-3/2)h. \qquad (A.17a)$$

Let us also define

$$T = t - (n-3/2)h. \qquad (A.17b)$$

With these new variables, and when (A.16) is introduced, (A.14) becomes:

$$\Delta F_k = \int_h^{2h} \mathrm{d}T\, \mathrm{e}^{-\lambda T}\left[\int_{T-h}^{T} (a_k X + b_k)\, \mathrm{d}x\right]. \qquad \text{(A.18)}$$

In order to obtain the values of a_k and b_k, we introduce the variable changes (A.17), we bring the expression (A.16) into (A.15), and perform the integration. We have then a linear relation in a_k and b_k. An other relation is obtained by reproducing the same kind of calculation as above in which k is replaced by $k-1$.

Finally, we have:

$$a_k = (G_k - G_{k-1})/h^2, \quad b_k = (3G_{k-1} - G_k)/h. \qquad \text{(A.19)}$$

Bringing these relations into (A.18) and performing the integration yields to:

$$\Delta F_k = \beta_{-1} G_{k-1} + \beta_0 G_k,$$

where

$$\beta_{-1} = (1/\lambda p)\,[1-(1+p)\,\mathrm{e}^{-p}]$$

$$\beta_0 = (1/\lambda p)\,[p-1+\mathrm{e}^{-p}], \qquad p = \lambda h. \qquad \text{(A.20)}$$

When $p \to 0$, β_{-1} and $\beta_0 \to h/2$.

When we take (A.20) into account, the recurrence relation (A.13) becomes:

$$F_k = F_{k-1}\,\mathrm{e}^{-\lambda h} + \beta_{-1} G_{k-1} + \beta_0 G_k. \qquad \text{(A.21)}$$

One has finally to calculate F_1 by the relation:

$$F_1 = \int_{h/2}^{3h/2} \mathrm{d}t\, \mathrm{e}^{-\lambda t} \int_0^t g(x)\, \mathrm{e}^{\lambda x}\, \mathrm{d}x. \qquad \text{(A.22)}$$

In order to evaluate F_1, we use the following binomial approximation:

$$g(x) = a_1 x + b_1 \qquad \text{(A.23)}$$

and the relations:

$$\int_{h/2}^{3h/2} g(x)\, \mathrm{d}x = G_1, \quad \int_{3h/2}^{5h/2} g(x)\, \mathrm{d}x = G_2,$$

from which one obtains:

$$a_1 = (G_2 - G_1)/h^2, \qquad b_1 = (2G_1 - G_2)/h. \qquad \text{(A.24)}$$

Finally, if we take (A.23) and (A.24) into account, (A.22) becomes:

$$F_1 = \beta'_0 G_1 + \beta'_1 G_2$$

where

$$\beta'_0 = p^{-2}\,[(p^2+p) - (1+2p)\,\mathrm{e}^{-p/2}(1-\mathrm{e}^{-p})]$$

$$\beta'_1 = p^{-2}\,[-p + (1+p)\,\mathrm{e}^{-p/2}(1-\mathrm{e}^{-p})]. \qquad \text{(A.25)}$$

For $p \to 0$ one obtains $\beta'_0 = 4h/3$, $\beta'_1 = -h/3$.

2) *Expression* (22) assumes the following form:

$$H(t) = \int_0^t g(x)\,(t-x)\exp[-\lambda(t-x)]\,\mathrm{d}x, \qquad \text{(A.26)}$$

which can be decomposed in the following way:

$$H(t) = \mathrm{e}^{-\lambda k} H(t-h) + kF(t-h) + \int_{t-h}^{t} g(x)\,(t-x)\exp[-\lambda(t-x)]\,\mathrm{d}x, \qquad \text{(A.25)}$$

where $F(t)$ is defined in (A.10).

We need the value of

$$H_k = \int_{(n-1/2)h}^{(n+1/2)h} H(t)\,\mathrm{d}t,$$

which becomes, if we take (A.25) into account:

$$H_k = \mathrm{e}^{-\lambda h}\,[H_{k-1} + hF_{k-1}] + \Delta H_k, \qquad \text{(A.26)}$$

where

$$\Delta H_k = \int_{(n-1/2)h}^{(n+1/2)} \int_{t-h}^{t} g(x)\,(t-x)\exp[-\lambda(t-x)]\,\mathrm{d}x. \qquad \text{(A.27)}$$

In order to calculate ΔH_k, one approximate $g(x)$ by the local binomial already used in the calculation of ΔF_k, and by proceeding in the same way as above. One finally obtains:

$$\Delta H_k = \gamma_{-1} G_{k-1} + \gamma_0 G_k,$$

where

$$\gamma_{-1} = (h^2/p^3)[p-2+(2+p)\,\mathrm{e}^{-p}],$$

$$\gamma_0 = (h^2/p^3)[2-(2+p+p^2)\,\mathrm{e}^{-p}]. \qquad \text{(A.28)}$$

For $p \to 0$, $\beta_1 \to h^2/3$ and $\beta_1 \to h^2/6$.

For the first term,

$$H_1 = \gamma'_0 H_1 + \gamma'_1 H_2$$

for $p \to 0$, $\gamma'_0 = 4h^2/3$ and $\gamma'_1 = 11h^2/12$.

If instead of $g(x)$, one uses the transient fluorescence $f(x)$ of a reference compound, one can see, according to (A.5) and (A.9), that one has also to perform convolutions of type (A.10) and (A.26). Therefore the algorithms developed in appendix 2 are also valid in this case.

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