DEVELOPMENT OF PICOSECOND TIME-RESOLVED TECHNIQUES BY CONTINUOUS-WAVE LASER AMPLITUDE MODULATION IV: SYSTEMATIC ERRORS

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Summary

Progress in the development of a dual-beam modulation technique for luminescence lifetime determinations is reported. The experimental details are described. Special attention is paid to the elimination of systematic errors. It is shown that, except in special cases, reliable absolute lifetimes are only available after determination of the phase delay caused by the wavelength-dependent photomultiplier transit time. Fortunately this turned out to be easy in our dual-beam experiment. Two independent methods of solving the problem caused by the photomultiplier transit time are discussed. The dual-beam feature of the experiment also enables the phase shifts caused by rotational diffusion to be determined easily.

The treatment is extended to multiexponential decays. An important result is that it is only necessary to measure the relative intensities of the a.c. This means that only a relative calibration of the detection apparatus is required.

1. Introduction

A time-dependent light forcing function $F(t)$ is required for any type of luminescence lifetime determination. The emission $E(t)$ must be registered in such a way that the transfer function $T(t)$ of the sample is reproduced with as little distortion as possible. The criteria determining the actual light forcing function which should be used in a particular type of measurement therefore depend on the problem under investigation and the technical feasibility of the measurement. However, technological developments make it necessary to reinvestigate and compare different approaches from time to time. In this context it is interesting to note that the first measurements of fluorescence lifetimes were made by phase delay techniques [1 - 4]. In the late 1930s Szymanowski [2] and Maerks [3] reported time resolutions down to 0.2×10^{-9} s with phase techniques. Later on this method was almost completely replaced by pulse fluorometry. What were the reasons for this?

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Phase fluorometry had three major disadvantages compared with the pulsed methods: systematic errors could occur, multiexponential decay events could not be resolved and the sensitivity was inadequate. It is possible that these difficulties could be resolved by the application of modern techniques and approaches. In view of our previous studies [5 - 7] and the results reported in this paper we believe that within the next few years modulation techniques will become of increasing importance in fluorescence decay measurements. The dual-beam method described here is superior to other modulation techniques.

2. Experimental arrangement and measurement procedure

A harmonic light forcing function $I \propto \sin^2 \omega t$ is used. The light emitted by a sample S as the result of a linear physical process such as scattering or emission is modulated by the same frequency as the exciting beam. It may be delayed by a phase shift Φ and its a.c. amplitude may be damped. The information about the time dependence of the processes under investigation is independently contained in the phase shift and the modulation depth. We have restricted ourselves to measuring the phase shift Φ between two modulated light signals. The need to compare two signals leads to the design of a dual-beam experiment in which one beam acts on a reference probe and the other on a sample. The phase difference between the two signals reflects differences in the time scale behaviour of all the processes involved. The measurement is therefore relative, and, as the reference probe can be chosen arbitrarily, it is applicable to different problems.

The following procedure is adopted to determine the phase difference. It was shown in ref. 5 that the phase difference can be obtained from measurements of three a.c. intensities: the reference intensity I_R, the sample intensity I_S and the sum I_B of I_R and I_S. After some mathematical transformations it is found that only the intensity ratios

$$\alpha = \frac{1}{2} \log\left(\frac{I_R}{I_S}\right)$$
$$\beta = \log\left(\frac{I_B}{I_S}\right) \qquad (1)$$

need be determined. The phase shift Φ_{exp} between the two signals is then given by

$$\cos \Phi_{exp} = 0.5 \times 10^{-\alpha}(10^{\beta} - 10^{2\alpha} - 1)$$

Three advantages of the dual-beam apparatus using this acquisition procedure should be mentioned.

(1) Absolute calibration of the apparatus is unnecessary. Relative calibration is sufficient as will be shown later.

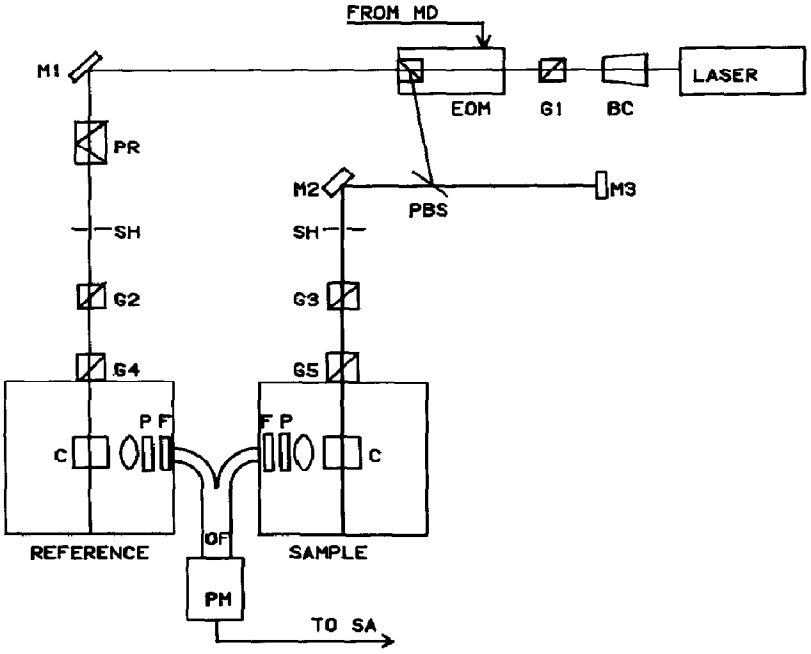

Fig. 1. The optical system (for details of the instrumentation see ref. 6): BC, beam collimator; P, sheet-type polarizer; G1 - G5, Glan prism polarizers; F, interference filter; EOM, electro-optic modulator; OF, optical fibre bundle; M1 - M3, mirrors; PM, photomultiplier; PBS, pellicle beam splitter; SH, mechanical shutter; PR, polarization rotator; MD, modulator driver; C, cuvette; SA, spectrum analyser.

(2) The time required to determine the phase difference is less than 20 s so that there are no stability problems.

(3) Since only the relative a.c. intensity is measured the sources of systematic error are reduced.

The precision of the phase determination from a series of measurements is better than 1 mrad; the accuracy predicted by the error estimation is better than 2 mrad. The latter value was confirmed in an experiment in which a known phase shift was introduced by changing the path length of one of the beams by a definite amount.

We now focus our attention on some details of the experimental arrangement (Fig. 1) including some changes to the apparatus described in ref. 6.

2.1. Modulation

An electro-optic modulator which allows any desired modulation frequency within the specified bandwidth to be used was chosen. When this modulator is combined with an RF spectrum analyser as the detecting unit frequency sweeping is possible. Phase shift determinations at various modulation frequencies are required to resolve multiexponential decays.

2.2. Dual-beam facility

An error treatment of the acquisition procedure (see ref. 5) shows that the phase shift between the two recorded signals should be close to π rad. This requirement is easily met by using the extraordinary beam of the modulator as the sample beam and the ordinary beam as the reference beam. The two beams exhibit an intrinsic phase difference π and are perpendicularly polarized with respect to each other. The use of these beams renders unnecessary the introduction of a long delay line which could cause serious mechanical stability problems. (The wavelength of a 30 MHz oscillation is 10 m.)

2.3. Polarization control

Orientational relaxation of the excited molecules or anisotropic samples may seriously perturb the lifetime measurements unless the polarization of both the exciting light and the detected light is controlled. The polarization of the exciting light is fixed by the Glan prisms G4 and G5, and the prisms G2 and G3 serve as continuous beam attenuators. As the two beams which leave the modulator have different polarizations, a polarization rotator PR is placed in one beam to make the two polarizations parallel. The emitted or scattered light passes through a sheet polarizer which can be rotated as desired.

2.4. Geometry

Each phase difference which is measured experimentally contains, apart from sample information, the path length difference of the two beams. This geometry-related phase difference $\Phi_{\Delta L}$ can be measured initially by making the sample and reference probe identical with respect to the chemical properties, the observation wavelength and the polarization. Care must be taken that the geometrical arrangement, which includes the paths of the exciting light, the optical elements in these paths, the probe illumination and light collection geometry, and the optical elements between the cuvette and the photodetector, is not changed in the subsequent measurements. It should be noted that nothing need be known about the contributions of these geometry effects; the only condition which must hold is that they remain constant during the measurements.

2.5. Reference probes

2.5.1. Lifetime measurements

The sample cuvette contains a solution of the dye whose luminescence lifetime is to be measured. The reference sample should thus be a light scattering solution as the scattering process can be regarded as infinitely fast. We found that an aqueous solution of Ludox (colloidal silica; particle diameter, about 15 nm) in a concentration such that the scattering efficiency is approximately the same as the emission efficiency of the luminescing sample (about 0.1%) is a suitable reference. The light collection geometry is exactly the same as that of the sample cuvette.

As with any other apparatus for lifetime measurement the different wavelengths of the scattered and emitted light lead to a systematic error caused by the photomultiplier transit time. The elimination of this error will be discussed in detail later.

2.5.2. Lifetime difference measurements

A standard sample of known lifetime can be used as the reference probe. The experiment then yields the lifetime difference between the two samples. The same procedure can be applied if only changes in the lifetime with some external parameter (*e.g.* temperature, viscosity, pH and concentration) are of interest. In this case no standard sample is required. This type of measurement is essentially free from the error discussed above as there is no wavelength difference between the two channels [7].

2.6. Detection (Fig. 2)

The emitted or scattered light is collected by the two arms of an optical fibre and guided to the photocathode of a photomultiplier. The fibre guarantees a reproducible and stable collecting geometry and a thorough mixing of the two signals.

Although we need only consider the a.c. part of the signal, care must be taken not to overload the photomultiplier by using too high a d.c. component which would lead to saturation effects and therefore to a systematic error.

The photocurrent from the photomultiplier is fed into the input of the spectrum analyser SA after blocking any d.c. component. The spectrum analyser measures the a.c. intensity of the signal at exactly the modulation frequency with a very narrow bandwidth of 30 Hz, thus suppressing most of the noise and any higher harmonics which might be present.

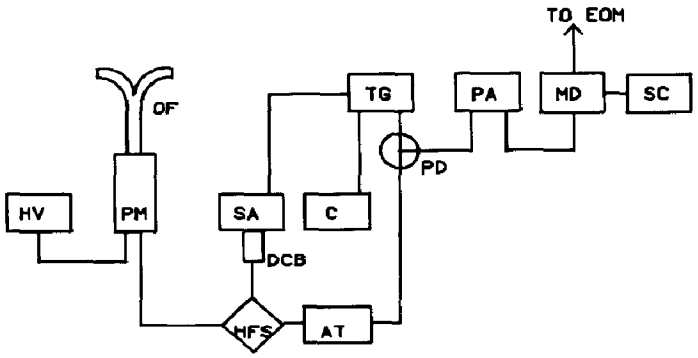

Fig. 2. Signal generation and detection equipment (for details of instrumentation see ref. 6): TG, tracking generator; HV, high voltage supply; PD, power divider, HFS, high frequency switch; PA, preamplifier; DCB, d.c. block; MD, modulator driver; SA, spectrum analyser; SC, oscilloscope; AT, step attenuator; EOM, electro-optic modulator; C, frequency counter; OF, optical fibre bundle; PM, photomultiplier.

2.7. Calibration

The performance of the apparatus depends on the accuracy of the relative intensity measurements performed using the spectrum analyser. The calibration of the dynamic range of this instrument is crucial. An electrical reference signal of the same frequency, which can be attenuated by a precision step attenuator, is applied to the spectrum analyser alternately with the signal from the photomultiplier. Scanning the intensity of this signal through the dynamic range of the analyser provides a step-by-step calibration curve. The procedure is fully automated.

3. Choice of the modulation frequency

The optimum frequency range for a given sample with lifetime τ is determined by two factors. The a.c. amplitude $A(\tau, \omega)$ of the emitted light is damped at high frequencies because the emitting sample cannot completely follow the excitation:

$$\frac{A(\tau, \omega)}{A(0, \omega)} = \frac{1}{(1 + \omega^2\tau^2)^{1/2}} \tag{2}$$

The amplitude ratio $A(\tau, \omega)/A(0, \omega)$ is shown in Fig. 3 for τ values of 10, 1 and 0.1 ns. The actual modulation frequency must be chosen such that the amplitude is not damped too much or there is a serious loss of signal-to-noise ratio. It should be noted that it is not advisable to use modulation frequencies above 200 MHz for decay times between 1 and 10 ns.

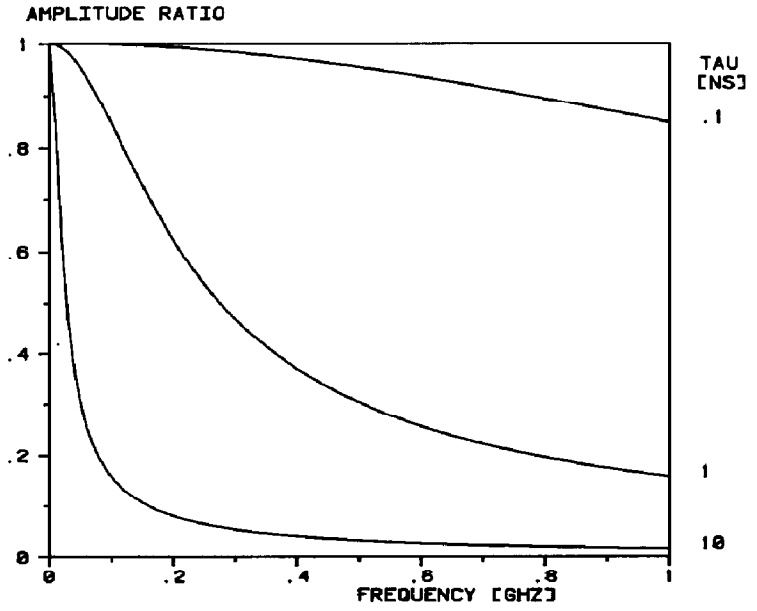

Fig. 3. Amplitude ratio $A(\tau, \omega)/A(0, \omega)$ (eqn. (2)) as a function of the modulation frequency from 0 to 1000 MHz for decay times of 10, 1 and 0.1 ns.

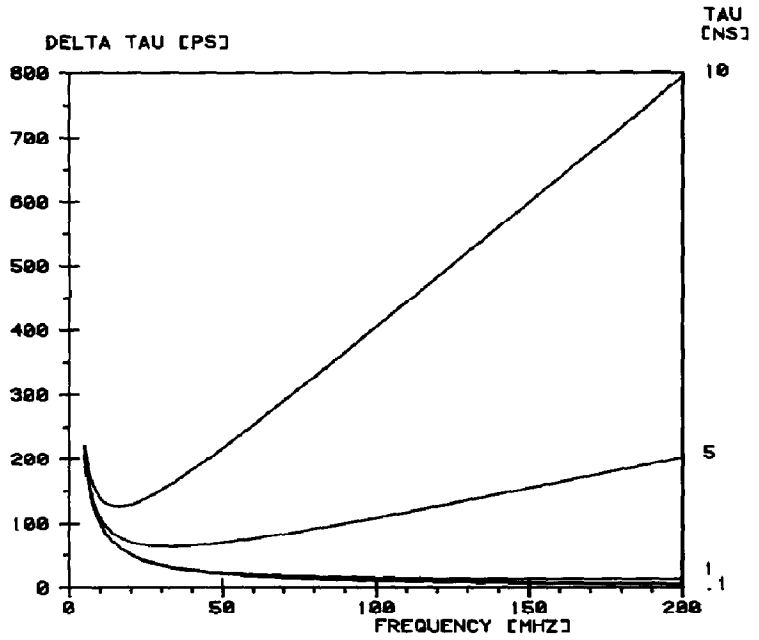

Fig. 4. Error propagation from the phase shift Φ to the lifetime for single-exponential decay. The error $\Delta\tau$ in the lifetime τ caused by the error $\Delta\Phi = 6.28$ mrad in the phase determination is shown as a function of the modulation frequency for τ values of 10, 5, 1 and 0.1 ns.

Every phase shift Φ which is measured between the two beams is subject to a certain error. When the lifetimes are calculated from the phase measurements, systematic errors in Φ may introduce large deviations from the true value of τ. It is therefore necessary to determine whether there is a frequency range for which the influence of this error on the lifetime calculation is minimized. The propagation of the error $\Delta\Phi$ to $\Delta\tau$ for a single-exponential decay is given by

$$\Delta\tau = \frac{\partial\tau}{\partial\Phi}\,\Delta\Phi = \frac{\tau^2\omega^2 + 1}{\omega}\,\Delta\Phi \qquad (3)$$

Figure 4 illustrates this relation for τ values of 10, 5, 1 and 0.1 ns and $\Delta\Phi$ = 6.28 mrad. This plot shows that there is in fact an optimum frequency range which lies between 10 and 100 MHz depending on τ. To improve the time resolution it is preferable to minimize phase errors rather than to increase the modulation frequency.

In general very high frequencies cause serious experimental problems. We have therefore restricted our studies to the region below 100 MHz.

4. Systematic errors and their elimination

Many papers [2 - 8] on modulation techniques have appeared since the initial work of Gaviola [1]. A fundamental problem, which was

recognized even in the very early papers, is the occurrence of systematic errors.

Let us suppose that we have a scatterer in the reference cell R and a fluorescent dye in the sample cell S. There will then be five contributions to the phase shift between the light collected from the reference and the light collected from the sample:

$$\Phi_{exp} = \Phi_S - \Phi_R$$
$$= \Phi_{\Delta L} + \Phi_{PM} + \Phi_\tau + \Phi_{rot} + \langle\Phi_N\rangle \quad (4)$$

We now have to extract from Φ_{exp} the phase shift Φ_τ due to the lifetime of the dye. The lifetime of a single-exponential decay is related to Φ_τ by

$$\tau = \frac{1}{\omega} \tan \Phi_\tau \quad (5)$$

Let us consider the various contributions to Φ_{exp} separately.

$\Phi_{\Delta L}$ is the phase shift caused by the path length difference ΔL of the two beams and is related to the speed c of light by

$$\Phi_{\Delta L} = \frac{\omega \Delta L}{c} \quad (6)$$

$\Phi_{\Delta L}$ can be measured if the sample and reference probes are identical as discussed in Section 2.4. Once this is done $\Phi_{\Delta L}$ can be eliminated from eqn. (4).

$\langle\Phi_N\rangle$ is the phase shift due to non-random noise sources such as the electro-optic modulator and other electronic components. In our experiment this phase shift can be estimated by measuring the noise level in the absence of light. The measurement procedure is described in detail in refs. 5 and 6. The value of $\langle\Phi_N\rangle$ for the signal-to-noise ratio in our experiment is about 2 mrad in the worst case.

Φ_{PM} is due to the photomultiplier transit time difference Δt_{PM}:

$$\Phi_{PM} = \omega \Delta t_{PM} \quad (7)$$

If the frequency of the light collected at the reference is different from the frequency of the light collected at the sample, Δt_{PM} is not zero. The photomultiplier transit time difference is a source of serious error in lifetime determination and must be eliminated. Whereas it is difficult to eliminate Δt_{PM} in pulse fluorometry it is relatively easy in the dual-beam technique [9]. If only relative lifetimes are to be measured, as is the case in rotational diffusion or quenching studies for example, the experiments can be performed in such a way that the values of Δt_{PM} cancel exactly. Two methods which are both based on the assumption that the photomultiplier response does not depend on the frequency, a condition which is obeyed by many photomultipliers operating below 100 MHz, can be used to eliminate Δt_{PM} in other cases.

The simpler method involves further assumptions concerning the properties of fluorescent dyes. Identical samples of a very dilute solution

of a fluorescent dye are placed in the reference and the sample cells which are then irradiated with light of the same polarization and wavelength. The fluorescence from the sample and the reference is collected under identical conditions of geometry and polarization. The dye chosen must be such that the luminescence decay follows a single-exponential function and that the radiative lifetime does not depend on the emitted wavelength. It should be noted that no precise information about the value of the lifetime is needed. If the fluorescence light is collected at the same wavelength λ_0 at the reference and the sample, the path length difference ΔL due to the photomultiplier transit time Δt_{PM} must be zero regardless of the modulation frequency. A negative path length difference $\Delta L_{PM}^{SR} = \Delta t_{PM} c$ is observed if the emission from the sample is measured at λ_0 and the emission from the reference is measured at $\lambda_0 + \Delta\lambda$. If our assumptions are correct, ΔL_{PM}^{SR} will not depend on the modulation frequency. This fact can be used to check the validity of the procedure. If, however, the emission from the reference is measured at λ_0 and the emission from the sample is measured at $\lambda_0 + \Delta\lambda$, $\Delta L_{PM}^{RS} = \Delta t_{PM} c$ will be positive. If there is no systematic error, $|\Delta L_{PM}^{SR} + \Delta L_{PM}^{RS}|$ will be less than the statistical error imposed by $\langle \Phi_N \rangle$. A typical result is shown in Table 1 where it can be seen that the systematic error introduced by the photomultiplier transit time is much larger than the accuracy of the experiment even for a wavelength shift $\Delta\lambda$ as small as 30 nm.

If we assume that it is possible to determine Φ_{rot} (Section 5), the only unknowns remaining in eqn. (4) are Φ_τ and Φ_{PM} which show different dependences on the modulation frequency ω. The second method of eliminating Φ_{PM} is based on this fact. The sum $\Phi^* = \Phi_\tau + \Phi_{PM}$ is available from experimental data. It is related to τ and Δt_{PM} by

$$\Phi^* = \arctan(\omega\tau) + \omega\, \Delta t_{PM} \qquad (8)$$

Φ_{exp} is measured at different modulation frequencies for a fixed experimental arrangement of the scatterer in the reference cell and the dye in the sample cell. The only assumption required is that dyes exist for which the luminescence decay follows a single-exponential function. The validity of this

TABLE 1

Photomultiplier transit time for an RCA 31'024 head-on tube measured using a solution of 8×10^{-7} mol rhodamine 6G l^{-1} in 90% ethanol–10% water

Observed wavelength (nm)		*Total path length L* (cm)	ΔL_{PM} (cm)	Δt_{PM} (ps)
Reference cell	*Sample cell*			
560	560	488.2 ± 0.4	0	0
560	590	490.5 ± 0.4	2.3	77
590	560	486.0 ± 0.4	−2.2	73

Excitation wavelength, 530.9 nm; modulation frequency, about 34 MHz; high voltage, −2000 V; full width at half-maximum of interference filter, 40 nm; Δt_{PM} includes a time delay which might be introduced by wavelength-dependent propagation of the light in the optical fibre.

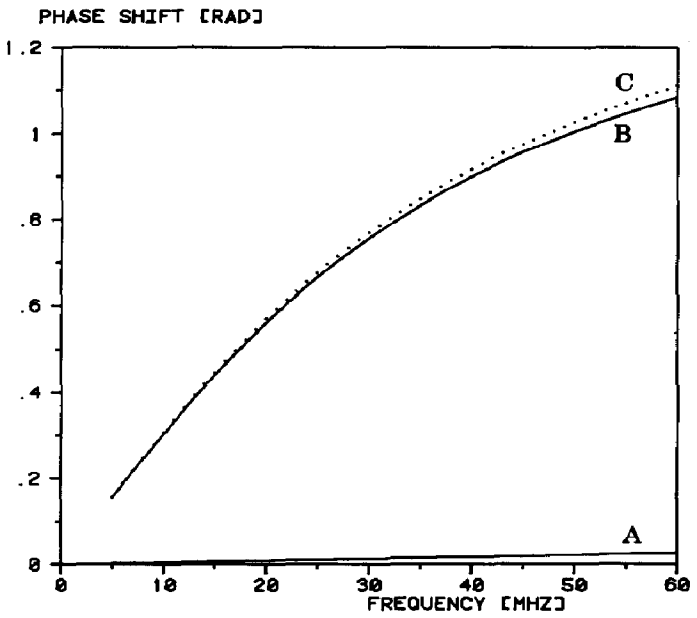

Fig. 5. Calculated frequency dependence of Φ_τ and Φ_{PM} assuming a single-exponential decay (τ = 5 ns; Δt_{PM} = 70 ps): curve A, Φ_{PM}; curve B, Φ_τ; curve C, Φ^*.

statement can again be checked by fitting eqn. (8) to the experimental data. If the model described by eqn. (8) adequately represents the behaviour of the system, the residuals of the fit can be assumed to be statistically distributed along the frequency axis.

The magnitude of the effect is illustrated in Fig. 5 where Φ_τ, Φ_{PM} and Φ^* calculated as functions of ω are shown for τ = 5 ns and Δt_{PM} = 70 ps. The value of Φ_{PM} at 50 MHz is 22 mrad, which is far greater than the accuracy of the phase determination.

If Φ_{PM} is not eliminated catastrophic systematic errors will result even for the determination of long lifetimes. We assume that τ is the true lifetime of the sample and that τ^* is the lifetime measured in the presence of a systematic error Δt_{PM}. Then the difference between τ^* and τ is given by

$$\Delta\tau = \tau^* - \tau = \frac{1}{\omega} \tan\{\arctan(\omega\tau) + \omega\,\Delta t_{PM}\} - \tau \qquad (9)$$

The error $\Delta\tau$ caused by Δt_{PM} is shown in Fig. 6 as a function of the modulation frequency ω for τ values of 10, 5, 1 and 0.1 ns. It should be noted that $\Delta\tau$ caused by Δt_{PM} = 50 ps becomes extremely large at long decay times. Therefore lifetime determinations using phase fluorometry can lead to erroneous results if Δt_{PM} is not eliminated. This is the major disadvantage of this type of measurement and may explain some contradictory results in the early literature. However, the advantage of this method is that the strong dependence of $\Delta\tau$ on Δt_{PM} can be used to determine Δt_{PM} with high accuracy because of the pronounced frequency dependence of $\Delta\tau$. Equation (9) and Fig. 6 show two other interesting properties of this type of modulation

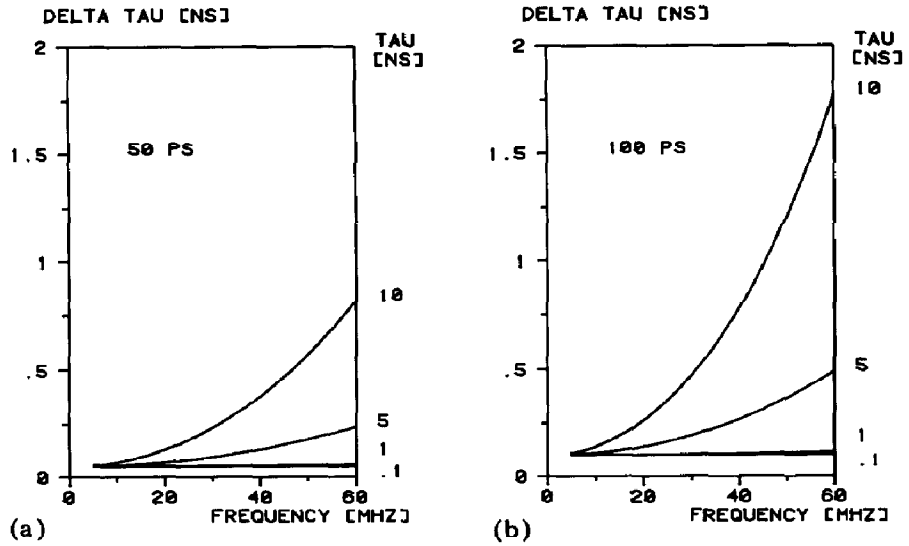

Fig. 6. Systematic error produced in the single-exponential lifetime by the photomultiplier transit time difference Δt_{PM} plotted as a function of the modulation frequency: (a) Δt_{PM} = 50 ps; (b) Δt_{PM} = 100 ps.

experiment. We can obtain results for the lifetime which are independent of the modulation frequency only if there is no systematic error and if the rate law can be described by a single-exponential function. This is an effective control for the accuracy of lifetime determinations. It should also be noted that the absolute errors introduced by Δt_{PM} are smaller for fast decays than for slow decays.

5. Rotational diffusion

The phase shift Φ_{rot} due to rotational diffusion is the only term in eqn. (4) which has not yet been discussed. We do not intend to explain the interpretation of this term here. All we want to show is that the accurate determination of Φ_{rot} is very simple in our dual-beam experiment. This is important for several reasons, one of which is that inaccurate values are obtained for τ if Φ_{rot} is neglected. Some results of measurements using polarized light are reported in Table 2 to demonstrate the determination of this phase shift. Δt_{rot} in Table 2 should not be confused with the rotational lifetime τ_{rot} which can only be determined by applying model assumptions such as the rotational diffusion of a sphere [10]. We do not intend to do this here. Δt_{rot} is just the time delay between the reference signal and the sample signal caused by rotational diffusion at an angle of 90° and a modulation frequency of 40 MHz. Δt_{rot} determined in this way is free of any systematic error.

TABLE 2

Influence of rotational diffusion in a solution of 8×10^{-7} mol rhodamine 6G l^{-1} in 90% ethanol–10% water

Sample cell [a]	*Reference cell* [a]	*Path length* (cm)	ΔL_{rot} (cm)	Δt_{rot} (ps)
Scatterer // + ⊥	Scatterer // + ⊥	421.3 ± 0.3		
Scatterer //	Scatterer //	420.9 ± 0.4		
Rhodamine 6G // + ⊥	Rhodamine 6G // + ⊥	421.1 ± 0.4	0	0
Rhodamine 6G ⊥	Rhodamine 6G //	429.0 ± 0.3	7.9	263

Excitation wavelength, 530.9 nm; modulation frequency, about 40 MHz; temperature, 25 °C.

[a] // means that only the component of the emitted light with parallel polarization relative to the strongly polarized exciting beam was collected, while ⊥ means that only the perpendicular component was collected. Measurements performed in the absence of the polarization analyser are indicated by // + ⊥.

6. Multiexponential decays

The treatment of non-exponential decays is only possible after the elimination of systematic errors. Otherwise large deviations from physically reasonable interpretations are obtained. We are now able to control the systematic errors and therefore we can investigate the problem of multiexponential decays. The extension to more complicated systems is straightforward. It is not a mathematical problem but depends on the accuracy of the experimental data. We keep the mathematics simple so that the procedure can easily be understood.

It can be shown that the experimental quantity E_{exp} must be determined for both single-exponential and multiexponential decays:

$$E_{exp}(\omega) = 0.5 \times 10^{-\alpha}(10^{\beta} - 10^{2\alpha} - 1) \tag{10}$$

where α and β are defined in eqn. (1). Therefore only the relative values α and β need to be measured for any type of decay and hence no absolute intensity calibration is necessary.

A value $E_{theor}(\omega)$ which is a function of the model parameters can be calculated for any model describing the processes involved. For a double-exponential decay in the absence of rotational diffusion this expression is given by

$$E_{theor}(\omega) = \frac{\cos(\Phi_{\tau_1} + \Phi_{\Delta L} + \Phi_{PM}) + \epsilon_{21}\cos(\Phi_{\tau_2} + \Phi_{\Delta L} + \Phi_{PM})}{\{1 + \epsilon_{21}^2 + 2\epsilon_{21}\cos(\Phi_{\tau_1} - \Phi_{\tau_2})\}^{1/2}} \tag{11}$$

where

$$\epsilon_{21} = q_{21}\left(\frac{1 + \omega^2\tau_1^2}{1 + \omega^2\tau_2^2}\right)^{1/2}$$

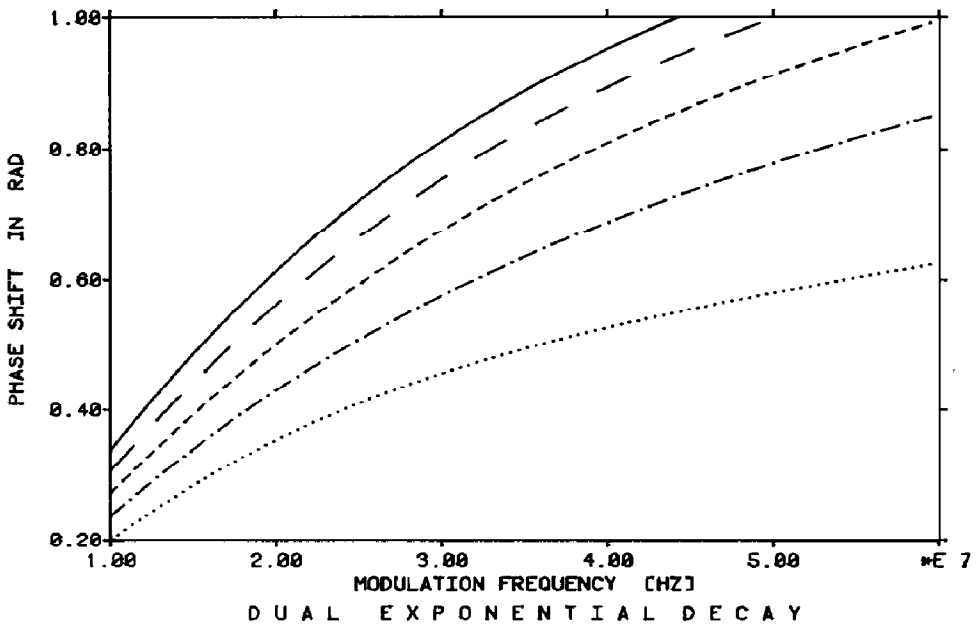

Fig. 7. Phase shifts caused by double-exponential decays calculated according to eqns. (11) and (12) for $\Phi_{\Delta L} = 0, \Phi_{PM} = 0$ and various values of (τ_1, τ_2): ———, (5.6 ns, 5.6 ns); — — —, (5.6 ns, 4.48 ns); - - - -, (5.6 ns, 3.36 ns); — · —, (5.6 ns, 2.24 ns); ·····, (5.6 ns, 1.12 ns).

q_{21} is the ratio of the quantum yield of the component with decay time τ_2 to that of the component with decay time τ_1. If the model applied is correct, the experimental value should be equal to the theoretical value for any modulation frequency:

$$E_{exp}(\omega) = E_{theor}(\omega)$$

The $E(\omega)$ values are related to the phase shift by

$$\Phi(\omega) = \arccos E(\omega) \qquad (12)$$

Thus E must lie in the interval $-1 \leqslant E \leqslant 1$.

In order to demonstrate the effect of the superposition of a second exponential decay on a single-exponential decay we show in Fig. 7 the calculated phase shift for five different combinations (τ_1, τ_2). The relative quantum yield q_{21} was assumed to be 0.9. The curve with the largest phase shift corresponds to a pure single-exponential decay (5.6 ns, 5.6 ns). The remaining phase shifts decrease in the order (5.6 ns, 4.48 ns), (5.6 ns, 3.36 ns), (5.6 ns, 2.24 ns) and (5.6 ns, 1.12 ns).

Since our measurements are accurate to approximately 10^{-3} rad there does not appear to be any fundamental difficulty in resolving exponential decays. We have investigated mixtures of different dyes with known lifetimes and quantum yields to obtain experimental data on the resolving power of our instrumentation.

The effect of a photomultiplier transit time Δt_{PM} of 70 ps is shown in Fig. 8 for three different combinations (τ_1, τ_2), *i.e.* (5.6 ns, 5.6 ns), (5.6 ns,

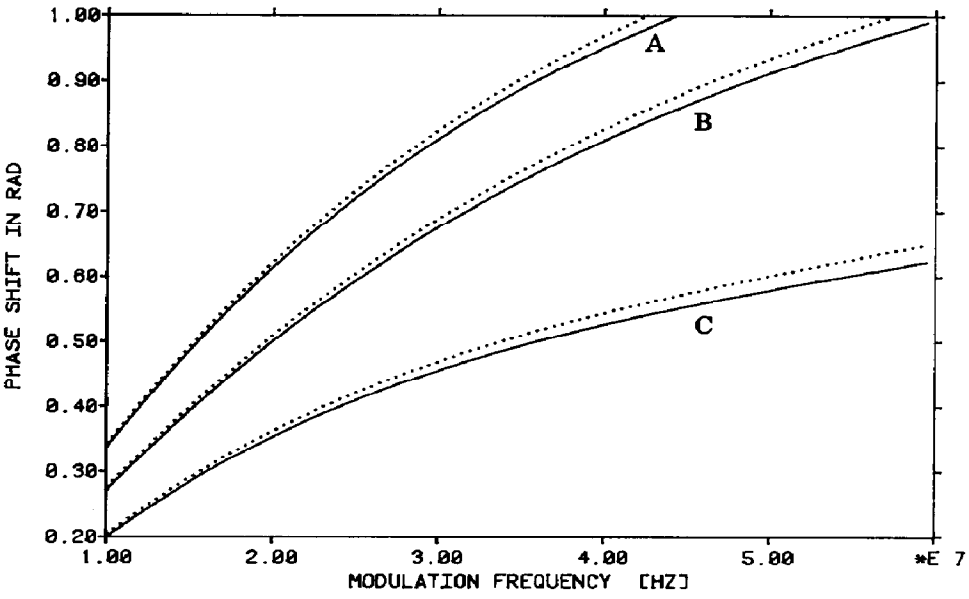

Fig. 8. Effect of Δt_{PM} on double-exponential decay events calculated according to eqns. (11) and (12) for $\Phi_0 = 0$ (·····, including $\Delta t_{PM} = 70$ ps; ——, $\Delta t_{PM} = 0.0$ ps): curves A, (5.6 ns, 5.6 ns); curves B, (5.6 ns, 3.36 ns); curves C, (5.6 ns, 1.12 ns).

3.36 ns) and (5.6 ns, 1.12 ns). We deduce from this figure that it is only possible to obtain a reliable interpretation of the experimental data after Δt_{PM} has been eliminated.

7. Sensitivity

There is no doubt that sensitivity was a problem in the original phase delay techniques. We have not yet established the limits of sensitivity in our experiment. Nevertheless the following data indicate that very sensitive measurements may be possible.

We need a signal-to-noise ratio of 40 dB for the a.c. power at the spectrum analyser. Because our noise level lies at approximately −120 to −125 dBm (Appendix A) we work with signal levels of the order of −80 dBm which corresponds to an a.c. power of 10 pW. Therefore the photomultiplier anode current required at an impedance of 50 Ω is approximately 0.5 μA. The actual photomultiplier currents are a factor of 2 - 4 larger because the exciting light is never completely modulated and the emitted light is damped.

These conditions were obtained with a solution of 8×10^{-7} mol rhodamine B l^{-1} in 90% ethanol–10% water with an exciting light flux of only 0.15 mW. The excitation wavelength was 530.9 nm and the emission was detected using an interference filter (588 ± 21 nm) and a photomultiplier supply voltage of −2 kV. If we take into account that the gain of the RCA 31'024 head-on tube at this voltage is approximately a factor of 20 lower

at 590 nm than between 350 and 500 nm at a voltage of −3 kV, these data demonstrate that our experimental arrangement is indeed very sensitive.

8. Conclusions

In ref. 7 we showed that the dual-beam feature of this experiment was an important step towards measuring small changes in luminescence relaxation kinetics. We have now demonstrated that this approach enables the problem due to the phase delay caused by the wavelength-dependent photomultiplier transit time to be solved easily. Therefore it is possible to use our method to measure absolute lifetimes and to study non-exponential decays. Although some problems remain, we now believe that this type of phase fluorometry can become a valuable supplement to pulse fluorometry. The next step will be to determine which problems are best suited to investigation by this method.

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Appendix A

The decibel (dB) is defined as the power ratio

$$10 \log_{10}\left(\frac{P_1}{P_0}\right)$$

Decibels above 1 mW (dBm) refers to $P_0 = 1$ mW at an impedance of 50 Ω; 0 dBm therefore corresponds to 1 mW. The relation of the power in decibels above 1 mW to the photomultiplier current i_{PM} in milliampères is given by

$$P = 20 \log_{10}\left(\frac{i_{PM}}{4.5}\right)$$