ISTITUTO NAZIONALE DI FISICA NUCLEARE
Laboratori Nazionali di Frascati

LNF-83/68(R)
20 Ottobre 1983

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A MULTIFREQUENCY CROSS-CORRELATION PHASE FLUOROMETER WITH PICOSECOND RESOLUTION USING SYNCHROTRON RADIATION

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ABSTRACT

The construction and operation of a cross-correlation phase and modulation fluorometer using the synchrotron radiation facility at the ADONE-Frascati electron storage ring is described. In the frequency domain the pulsed source gives a large series of equally spaced harmonic frequencies. Use of cross-correlation techniques in conjunction with a high repetition rate pulsed light source permits one to isolate one harmonic frequency from the adjacent frequencies with high precision. The cross-correlation frequency required for the analysis of the phase delay and modulation ratio is obtained using two coupled frequency synthetizers, one of which drives the radiofrequency cavity of the storage ring and the other which modulates the response of the

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photomultipliers used for the signal detection. The accuracy, reproducibility and sensitivity of the instrumentation have been determined experimentally. A study of measurement artifacts related to the color error of the photomultipliers has been carried out and no sensible color error was detected. Tryptophan emission was investigated in different pH conditions and at several excitation and emission wavelengths. Results of experiments on energy transfer between tyrosine and tryptophan in bovine serum albumin (BSA) and between tyrosine-tryptophan and bis-anilino-naphthalenesulfonate (bis-ANS) adsorbed to BSA are also presented. Energy transfer causes a definite lengthening of the measured phase angle due to the delayed emission of the acceptor excited through the donor relative to direct excitation. Lifetime measurements have been performed in color center of NaF excited in the various absorption bands.

1. - INTRODUCTION

Time resolved fluorescence emission is one of the basic methodologies used to study spectroscopic properties of excited states of molecules and solids. A pulsed light source as provided by synchrotron radiation is particularly appropriate for excitation because of the possibility of continuously varying the wavelength range and because of the short duration and high repetition rate of the pulses. Generally the fluorescence emission after a pulse excitation is measured in the time domain using the popular technique of time correlated single photon counting (SPC). We may, however, inquire whether phase fluorometry can be used in conjuction with a high repetition rate pulsed light source and specify the advantages that result from the application of the harmonic method. To this end we compare in this section the two alternative techniques.

Phase fluorometry has been used extensively in the past decade for the measurement of fluorescence lifetime of organic molecules[1, 2]. The advantages of phase fluorometry compared to SPC and other pulse methods are the high accuracy of the lifetime determination, the ease of measuring subnanosecond lifetimes and the rapidity of data collection. Picosecond resolution with phase fluorometry can be obtained using conventional light sources and acousto-optic modulators[3]. Direct differential phase measurements have been used to detect small variations in lifetimes due to solvent relaxation[4] and to study fast rotations of fluorophores utilizing polarization techniques[5].

Conventional phase fluorometers use a high intensity arc lamp or a continuous wave laser[3, 6-8]. The continuous light source is sinusoidally modulated by an acousto-optic modulator or electro-optic device. The frequency of modulation of the light intensity ranges from 1 to 200 MHz depending on the particular experimental arrangement used. In the past the main criticism concerning phase fluorometry was that multiexponential or non-exponential decays could not be analyzed. This criticism is valid only if phase data at a single modulation frequency are considered. If a wide set of

modulation frequencies are available, heterogeneous emission can, in fact, be accurately analyzed[3].

The impulse response and the harmonic response are mathematically related for systems which do not exhibit non-linear (saturation effects, etc.) behavior. The frequency response of the system can be obtained from the pulse response through the use of Fourier transforms. Complete equivalence requires knowledge of the system's response over a wide time and frequency range. The time domain response of a fluorescence system is defined when the amplitude of the emission, I(t), is measured from times immediately after excitation of the system by an ideal pulse of light (a delta function) to infinitely long times when the system has returned to the original state. I(t) can be either a single exponential decay, a sum of exponential decays or any other time function. In the frequency domain the fluorescence response is determined when the phase delay ϕ and the modulation ratio M of the emission with respect to an ideal sinusoidal excitation are measured at all frequencies. The expressions relating the time response to the frequency response are

$$G(\omega) = \int_0^\infty I(t) \cos(\omega t)\, dt , \tag{1}$$

$$S(\omega) = \int_0^\infty I(t) \sin(\omega t)\, dt . \tag{2}$$

The relationships between S and G and the observable phase and modulation values at a given frequency are given by

$$\phi = \tan^{-1}(S/G) , \tag{3}$$

$$M^2 = S^2 + G^2 . \tag{4}$$

Knowledge of only one portion of the frequency and time domain and excitation with non-ideal pulses renders impossible a direct comparison of the data obtained for the impulse and harmonic response since the integrals in equations (1) and (2) cannot be evaluated exactly. Fortunately, we require to know only the parameters associated with the time response of the system, e.g., the characteristic exponential decay time, τ, in the case of a single exponential decay. The same parameter can be obtained from the frequency response without transformation to the time domain. Our purpose, then, is to obtain the parameters of the decay rather than the decay itself. We can devise a procedure in the frequency domain in order to analyze a given fluorescence system. Using a sinusoidally modulated light source at an angular frequency ω we can obtain

the values of phase and modulation at that frequency and subsequently vary the frequency over a significant interval. We can then calculate the analytical response of the system (the model) directly in the frequency domain, i. e., we relate the observable phase and modulation values to the parameters characteristic of a given system. Finally, we can perform a fit, in the frequency domain, of the desired parameters to the experimentally determined values of phase and modulation. The derived parameters must coincide with those obtained in the time domain because of the mathematical equivalence of the two methods. Only at this stage can we make a meaningful comparison of the parameters obtained from the impulse and harmonic methdos.

However, the mathematical equivalence of impulse and harmonic response data does not mean that practical differences between the methods used to obtain the time and the frequency information do not exist. These differences depend on the particular procedure employed to collect data. In the following comparison we assume that the well established SPC method is utilized in the time domain. One important difference, seldom mentioned, concerns the fact that the impulse response technique does not permit the analysis of photons originating over the complete time domain. The time domain data is in essence truncated and obliged to be between somewhat arbitrary limits in addition to falling into time bins of finite width. For example, in the SPC technique one is permitted to observe at best only one photon per exciting pulse (to avoid statistical pileup effects this limit is, in fact, reduced) whereas in the harmonic response approach all the photons contribute to the measured signal. This latter fact confers a sensitivity to the harmonic method which compares favorably with that of SPC contrary to a prevalent belief.

We may note that very high frequencies are not necessarily required for the measurement of nanosecond decays with phase fluorometry. The errors in the phase and modulation measurements show a minimum at a given frequency[9] which depends upon the lifetime, e. g., one nanosecond may be most accurately measured near 150MHz but high precision can still be obtained at much lower frequencies. An arbitrary increase on the frequency will eventually lead to an increase in the error.

Another aspect of the comparison of the impulse and harmonic approach concerns the time required for a measurement. Using traditional flashlamp light sources collection of pulse data of reasonable precision may take many minutes while the actual computer analysis of the results requires an additional period. In contradistinction phase and modulation data at a single frequency can be obtained in a few seconds while data at several frequencies can be collected in a minute or so. Data analysis is sufficiently rapid to be performed virtually in real time with the data collection which

allows the experimentalist to monitor a situation closely and to contemplate kinetic life time studies on systems with rate constants in the range of seconds.

Furthermore, in the frequency domain the use of phase sensitive detection permits one to record directly the individual spectra of two components with different life times and spectra[9-16]. Such a direct recording is not obtainable at present using the impulse method (note that "time resolved" spectra obtained using single-channel--pulse-height analysers to set time windows roughly approximate the true time resolved spectra but are not equivalent to the direct phase sensitive recordings). In appendix I we give a more detailed comparison between pulse and phase methods.

Once we have illustrated the general features of the harmonic method, we must describe how to adapt this method to a pulsed light source. In section 2 we describe the basis of the method and in section 3 the experimental approach used.

2. - PHASE FLUOROMETRY AND SYNCHROTRON RADIATION (SR)

2. 1. - General

Gratton and Lopez-Delgado[9] suggested that a high repetition rate pulsed light source could be employed for multifrequency phase fluorometry instead of a sinusoidally modulated source. The advantage of using a high repetition rate pulsed light source consists of having a large number of modulation frequencies contemporaneously. A typical high repetition pulsed light source is the synchrotron radiation emitted in storage rings. Consider the time characteristic of the radiation emitted by a storage ring such as ADONE at the INFN National Laboratories in Frascati. Electrons travel in bunches and, when the ring is operating in the single bunch mode, the interval between two pulses is 350 nsec. The shape of the light pulse is approximatly Gaussian with a FWHM of about 2 nsec (Fig. 1a).

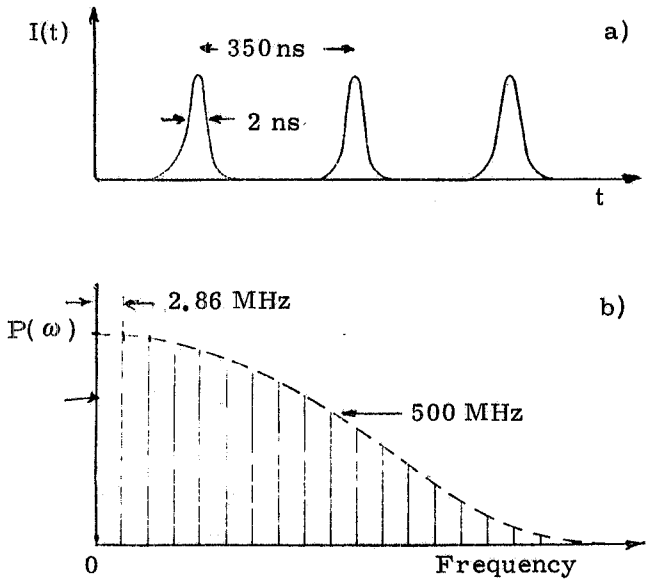

FIG. 1 - a) Schematic representation of the light pulses emitted by the storage ring when operating in the single bunch mode. b) The power spectrum of the pulse train shown in a).

The corresponding frequency transform consists of a set of harmonic frequencies 2.856167 MHz apart. This frequency set (a comb function) has a gaussian envelope with a half width of 500 MHz (Fig. 1b). This source is ideal for multifrequency phase fluorometry. The major problem is selecting a particular harmonic frequency while rejecting adjacent frequency components. Cross-correlation methods provide a simple, yet powerful technique for selecting a single harmonic frequency. Furthermore, using synchrotron radiation, a continuously variable wavelength range is available. The intensity in the near ultraviolet region of the spectrum is comparable to or greater than that from conventional arc lamps. Another interesting characteristic of synchrotron radiation is that all wavelengths are emitted simultaneously. This property can form the basis of a direct differential measurement on emission resulting from excitation at different wavelengths.

Some preliminary applications of phase techniques in conjuction with synchrotron radiation have been reported[17, 18]. In these reports, however, only one or two harmonic frequencies were employed and the cross-correlation technique was not utilized.

2.2. - Consideration of the signal available at different harmonics

In the introduction we pointed out that conventional phase fluorometry makes better use of the light intensity than single photon counting. The analysis of how the light intensity is used in phase fluorometry for a pulsed light source is interesting. In Fig. 1b we show the power spectrum of synchrotron radiation emitted at ADONE storage ring, which is wavelength independent. The intensity of the component at zero frequency (the average value) has approximately the same intensity as the component at 2.86 MHz. The intensity of the next component decreases only slightly. At about 500 MHz the intensity has dropped by a factor of two. The DC component represents the average light intensity. All the photons emitted contribute to this component and the measurement of the average light intensity is, of course, performed using the full light intensity. By the same argument all photons contribute to the first harmonic frequency, because the intensity of the first harmonic is approximately the same as that of the DC component. Again, the measurement at the n-th harmonic is performed using the full light intensity. (In the time domain this situation is equivalent to considering the light pulse as being composed of the sum of all its photons, each photon in the pulse contributing to the pulse shape). To conclude, in multifrequency measurements the average signal measured at the n-th harmonic frequency has approximately the same intensity as the complete fluorescence signal, the sensitivity being the same at all frequencies.

2. 3. - Application of the cross-correlation technique to synchrotron radiation

A great improvement in phase fluorometry was the introduction of cross-correlation techniques. This method was accurately described by Spencer and Weber[6]. The advantage of using cross-correlation resides mainly in the very high sensitivity, very low noise and extremely high accuracy in the determination of the phase delay and modulation ratio. In the following discussion we recall briefly the operational principles of a cross-correlation phase fluorometer using a sinusoidally modulated light source. In part 2.4 we develop the general equations for phase fluorometry using a pulsed light source.

For sinusoidal modulated excitation at ω frequency the emitted light intensity can be described by

$$F(t) = F_o\left[1 + M_F \sin(\omega t + \phi)\right] , \tag{5}$$

where F_o is the average fluorescence intensity, M_F the modulation ratio and ϕ the phase delay. In the cross-correlation method the detected emission F(t) is multiplied by a sinusoidal signal of frequency

$$C(t) = C_o\left[1 + M_c \sin(\omega_c t + \phi_c)\right] \tag{6}$$

where C_o is the average value of the multiplying function, M_c the modulation ratio and ϕ_c the phase delay. The resulting product signal is the new function

$$V(t) = F_o C_o\left[1 + M_F \sin(\omega t + \phi) + M_c \sin(\omega_c t + \phi_c) + M_F M_c \sin(\omega t + \phi)\sin(\omega_c t + \phi_c)\right] . \tag{7}$$

Using trigonometric relationships the last term in eq. (7) can be rearranged to

$$\frac{M_F M_c}{2}\left[\cos(\omega t + \omega_c t + \phi + \phi_c) + \cos(\Delta\omega t + \Delta\phi)\right] \tag{8}$$

where $\Delta\omega = \omega_c - \omega$ and $\Delta\phi = \phi_c - \phi$. If ω_c is chosen to be very close to ω eq. (7) contains a constant term plus a term of frequency ω plus a term of frequency 2ω and, finally, a term of frequency $\Delta\omega$. This last term contains all the phase and modulation information of the original fluorescence signal and can be totally filtered from the remaining terms.

For synchrotron radiation the light intensity cannot be approximated by a pure sinusoidal signal. Furthermore in real systems the electronic signal used for the cross-correlation product is also not purely sinusoidal[8]. The effect of the harmonic content of the F(t) and C(t) signals is discussed next.

2. 4. - Analysis of the harmonic content

For a pulsed source the exciting light can be approximated by the series:

$$I(t) = \sum_k I_k \sin(k\omega t + dk) \qquad (k = 0, 1, 2, \ldots) \qquad (9)$$

where I_k is the amplitude at frequency $k\omega$, dk a phase shift characteristic of each frequency and ω the base repetition frequency. The modulation of the exciting light is defined as the ratio of the intensity at a given frequency to the intensity at zero frequency. Each individual harmonic $k\omega$ is attenuated and phase shifted by the fluorescence sample. The signal detected by the photomultiplier due to the emission has the form:

$$F(t) = \sum_k F_k \sin(k\omega t + \phi_k) . \qquad (10)$$

The cross-correlation signal for the modulation of the photomultiplier response can be described by a similar expression:

$$C(t) = \sum_l C_l \sin(l\omega_c t + \phi_{c,l}) \qquad (l = 0, 1, 2, \ldots) . \qquad (11)$$

The waveform at the output of the photomultiplier can be described by the following relationship:

$$V(t) = \sum_k F_k \sin(k\omega t + \phi_k) \sum_l C_l \sin(l\omega_c t + \phi_{c,l}) . \qquad (12)$$

The average value of V(t) is given by the term at zero frequency:

$$\langle V \rangle = F_o C_o . \qquad (13)$$

This voltage constitutes the DC signal. Note that the DC signal depends on C_o.

In order to calculate the harmonic content of V(t) consider the term at the lowest frequency. If ω_c is very close to ω the lowest frequency term corresponds to $\Delta\omega = \omega_c - \omega$ and is obtained for $k = 1$ and $l = 1$

$$F_1 C_1 \sin(\omega t + \phi_1) \sin(\omega_c t + \phi_{c,1}) . \qquad (14)$$

This term is the only one giving a frequency $\Delta\omega$. All other combinations of k and l give a frequency $2\Delta\omega$ or greater. In section 3 we discuss how to filter the term of frequency $\Delta\omega$ with respect to $2\Delta\omega$. This term at $\Delta\omega$ is called the AC signal. The modulation, i. e. the AC/DC ratio is given by:

$$M_F = F_1 C_1 / F_o C_o . \qquad (15)$$

In practice we measure the modulation of the fluorescence with respect to the modu-

lation of a scatter solution, a quantity called the modulation ratio. This ratio depends only on the demodulation of the fluorescence signal and not on the details of the electronics.

3. - DESCRIPTION OF THE EXPERIMENTAL SETUP

3. 1. - Cross-correlation electronics

An improvement on the original Spencer and Weber cross-correlation phase fluorometer was recently reported[8]. In this later version a continuously variable frequency range is available. Virtually the same method of obtaining the cross-correlation frequency was applied to synchrotron radiation. The interested reader can find in ref. (8) the operational principle of the multifrequency phase fluorometer as well as a discussion of the possible instrumental artifacts. With reference to Fig. 2, the light source is the synchrotron radiation. The repetition frequency of the synchrotron radiation is controlled by a frequency synthetizer (Hewlett Packard mod. 3525A). This synthetizer generates the driving frequency for the radiofrequency cavity of the storage ring at 8. 568500 MHz. The frequency for the cross-correlation is produced by a second frequency synthetizer (Rockland 5600A) which uses the same crystal oscillator than the Hewlett-Packard synthetizer. The output of this synthetizer which is phase coherent with the radiofrequency of the storage ring can be varied in order to obtain a frequency which is equal to the fundamental frequency, or to one of the harmonic components, plus 24 Hz. The small difference, 24 Hz, is the cross-correlation frequency, $\Delta\omega_c$. The output of the synthetizer is amplified by an rf power amplifier (ENI 503L), split

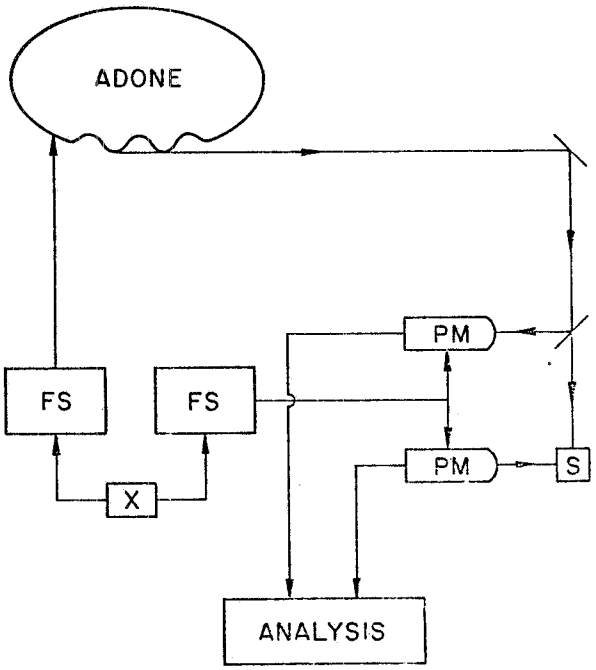

FIG. 2 - Block diagram of the instrument. ADONE : storage ring at Frascati; PM : Hamamatsu R928 photomultiplier; S: fluorescence sample; FS: frequency synthetizer; X: crystal oscillator.

into two equal parts and applied to the last dynode of the reference and sample photomultipliers. The detail of the photomultiplier circuit are described in ref. (8).

For the synchrotron radiation setup the cross-correlation frequency at 24 Hz was extremely stable in both the short and long term. Ower a period of two hours the maximum deviation of the cross-correlation frequency was less than 0. 01 Hz. This stability implies that the oscillations of the electron beam in the storage ring are time averaged and do not influence the phase delay and modulation ratio measurements.

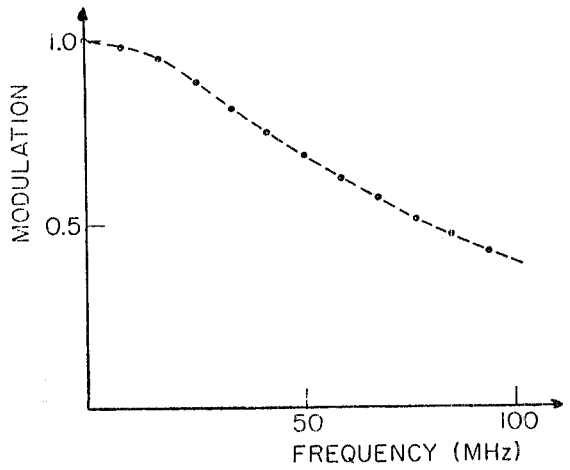

FIG. 3 - Relative modulation measured at harmonic frequencies below 100 MHz.

Due to the limited range of the frequency synthetizer utilized, the phase delay and the modulation ratio were measured at each harmonic frequency only up to 100 MHz. In principle higher frequencies can be used but the real limit then becomes the frequency response of the detector. The photomultiplier employed (Hamamatsu R928) has a maximum frequency of operation of about 200-300 MHz. In Fig. 3 we report the measured modulation ratio as a function of the frequency for a scattering solution. Frequencies as high as 100 MHz are attenuated only one half with respect to the fundamental demonstrating that high frequency measurements are feasible. A faster photomultiplier would permit access to a wider frequency range.

3. 2. - Electronic detection system

The electronic module for signal acquisition and analysis was built from commercial components. In Fig. 4 we report a block diagram of the electronics. The output of the two photomultipliers are analysed separately by two identical channels. In the following we describe the operation of only one of the channels.

After an initial amplification stage (A1) the signal is separated into DC and AC components. The DC component is integrated (I) in order to generate a DC signal proportional to the average intensity of the detected signal. The AC component is sent to an amplifier (A2) and filtered by a band pass active filter (F) to select the 24 Hz component. The output of the filter is rectified (R) and integrated (I) to produce a con-

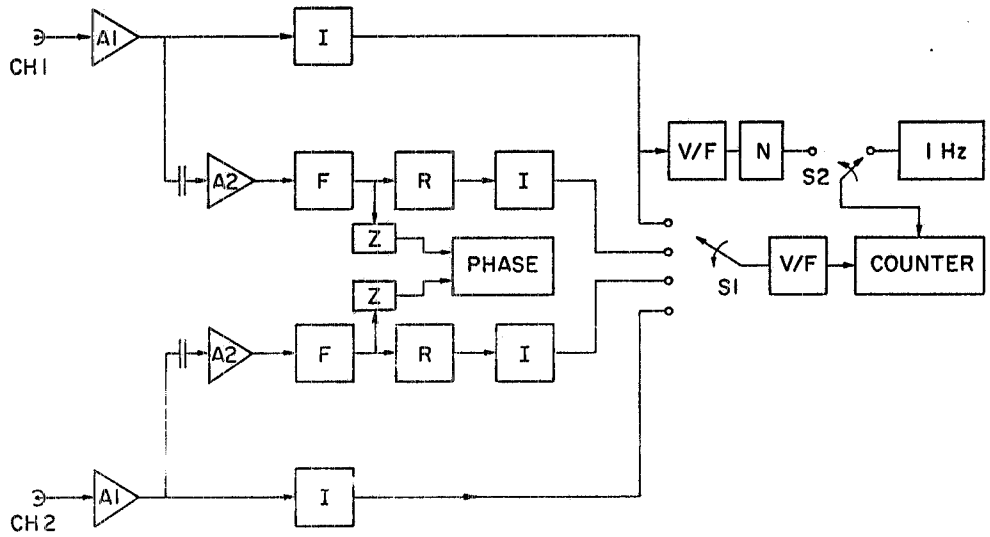

FIG. 4 - Block diagram of the electronic detection system. A1 and A2: low frequency amplifiers; I: integrators; F: band pass filters at 24 Hz; R: rectifiers; Z: zero-crossing detectros; V/F: 100 KHz voltage-to--frequency converters.

tinous voltage proportional to the AC component. The DC and AC parts of each channels are continuously monitored by four digital voltmeters (dual slope analog-to-digital converters 3 1/2 digits). These voltmeter readings are not utilized for data acquisition but only for rapid inspection of the signal levels. Accurate measurements of the signal are carried out by a precision integrating-digital-voltmeter (DVM) consisting of a voltage-to-frequency converter (V/F) and counting electronics. A switch (S1) selects the signal (AC or DC) input to the counter which is timed by a 1 MHz clock (Fig. 4). The resolution of the DVM is 0.1 mV per 1 second integration time. The digitized DC or AC components are presented on a six digit display. The ratio of the AC to the DC part can be obtained by replacing the 1 MHz clock by the output of the V/F converter utilized for the DC component. This mode of operation, the ratio mode, is selected by the switch S2. In the ratio mode the resolution of the modulation measurement is 1/10000.

The phase difference between the reference and the sample signal is measured by a digital phasameter (Fig. 4). The output of the two active filters (channels 1 and 2) are sent to zero-crossing detectors (Z), where two square-waves are produced. The positive going edge of these square-waves are used to start and stop the phase counter. The input of the phase counter is the 1 MHz clock. The content of the counter, in microseconds, is proportional to the phase difference between the signals from the two channels and is monitored by a six digit display (the phase display). The reso

lution for phase measurements is 1 microsecond which corresponds to an angular resolution of about 0.01°. One may integrate the phase readings for effective integration periods of 1, 2 or 8 seconds.

3. 3. - Optical arrangement

The fluorometer utilizes the visible and UV radiation available from the VUV beam line of the PULS Laboratory at ADONE storage ring[29]. The optical path is partially in the vacuum system and after a sapphire port travels in air.

The light beam is focused by a quartz lens onto the entrance slit of a holographic grating monochromator (SLM mod. 320A). The grating has 1500 lines/mm and is maximized at 300 nm. After dispersion in the monochromator, the light enters an optical module (SLM OP450) equipped with a rotating turret to permit facile exchange between the sample and reference. Standard fluorescence cuvettes as well as solid samples can be employed. For liquid samples a circulating thermostatic bath can be used to control the temperature. Solid samples are mounted on a cryostat cooled at liquid nitrogen temperature.

The emission is collected with a large aperture lens and focused onto a photomultiplier (Hamamatsu R928). A quartz beam splitter is placed in the optical path prior to the sample to direct a fraction of the exciting light to a reference photomultiplier (Hamamatsu R928) which measures the intensity and phase of the excitation signal. Generally the emission is viewed either through a suitable filter or an analysing monochromator. Calcite prism polarizers can be interposed in the excitation and emission light paths. The spectrum of the exciting light (200-750 nm) measured with our monochromator/photomultiplier system is reported in Fig. 5.

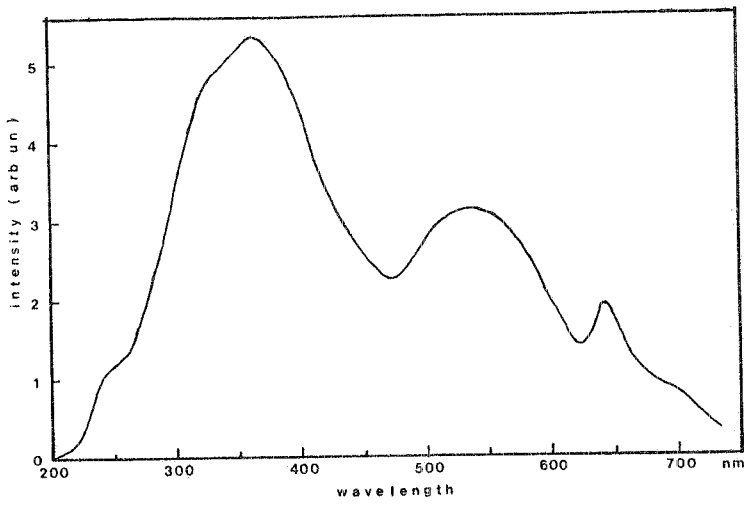

FIG. 5 - Light intensity at the output of the excitation monochromator SLM 320A) as a function of the photon wavelength. The slitwidth was 0.5 nm.

4. - APPLICATIONS

4. 1. - Instrument performance tests

4. 1. 1. - Rationale

A large number of measurements were performed with the aim of testing the resolution, sensitivity, accuracy and reproducibility of the lifetime determination. Well characterized fluorophores with lifetimes ranging from 100 psec to 30 nsec were studied utilizing the entire attainable frequency range.

4. 1. 2. - Short lifetimes : accuracy

A solution of p-terphenyl in cyclohexane was excited at 280 nm (8 nm slits) and the emission was observed through a Corning 0-52 filter. The solution was thermostated at 20°C but was not degassed; the optical density (OD) at 280 nm was 0.1. In Fig. 6 the phase and modulation values corresponding to this emission are plotted.

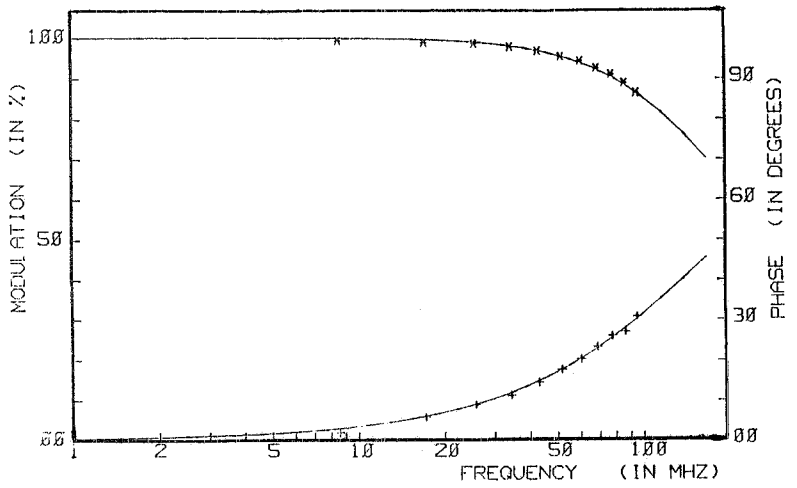

FIG. 6 - Multifrequency phase (+) and modulation (*) data for p-terphenyl in cyclohexane at 20°C. Solid lines correspond to the best fit using a single exponential component $\tau_o = 0.973 \pm 0.027$ nsec.

The solid lines correspond to a fit using a single exponential with $\tau = 0.973 \pm 0.027$ nsec. The fit is obtained using the non-linear least-squares routine described by Jameson and Gratton[19]. We note that i) the fit to a single exponential is quite good; ii) the measured lifetime corresponds to the literature value[28]; and iii) frequency dependent systematic errors do not occur.

4. 1. 3. - Long lifetimes : color errors

The lifetime of a solution of DENS (2. 5 diethylaminoethylnaphthalenesulfonate) in water was measured. The excitation wavelength was 360 nm and the emission was

measured using a cut-off filter ($\lambda_{EM} > 420$ nm). The purpose of this experiment was to study color delays. Jameson and Weber[20] have shown that the color delay will be amplified using a long lifetime fluorophore. At high modulation frequency a large apparent value of lifetime can be measured using phase data if there is a lengthening of the phase due to color effect. In Table I we report the measured values of lifetime using phase and modulation data for DENS (τ_o = 30 nsec). No apparent lengthening is detected. On the contrary a small shortening of the phase lifetime is observed. This result is consistent with a very weak component of short lifetime. The data were analyzed using a least-square routine[19] for one and two components.

TABLE I - Lifetimes for DENS in water : $\lambda_{EM} > 420$ nm ; τ^P and τ^M in nsec.

Freq. (MHz)	λ_{EXC} 240 nm		λ_{EXC} 360 nm	
	τ^P	τ^M	τ^P	τ^M
8.568	--	--	30.572	30.351
17.137	--	--	29.401	30.315
25.705	30.417	30.395	29.289	30.304

4.1.4. - Reproducibility

To test the reproducibility of the measurements we performed a series of lifetime determination on solutions of NADH. At two hour intervals fresh solutions were prepared and the lifetime was measured at 94.253 MHz. In Table II we report the results of this study. The errors shown in Table II correspond to the standard deviation of a series of six successive lifetime determinations for each sample. All phase lifetime determinations fall in a range of 6 psec. Normal operating conditions were used: no attempt was made to work in the best instrumental condition (maximum ring current, longer averaging time, etc.).

TABLE II - Lifetimes for NADH in water : λ_{EXC} 340 nm ; $\lambda_{EM} > 420$ nm (Freq. = 94.253 MHz).

Sample no.	Phase lifetime	Modulation lifetime
1	464 ± 11 psec	494 ± 14 psec
2	470 ± 5 psec	499 ± 9 psec
3	470 ± 5 psec	478 ± 18 psec
4	471 ± 10 psec	474 ± 20 psec

4. 1. 5. - Sensitivity

To test the sensitivity of the instrument the lifetimes of two weakly emitting fluorophores, bis-ANS and bis-TNS (bis-toluidyl-aminonaphthalenesulfonate)[21], were measured. The phase and modulation data obtained for bis-ANS are reported in Fig. 7.

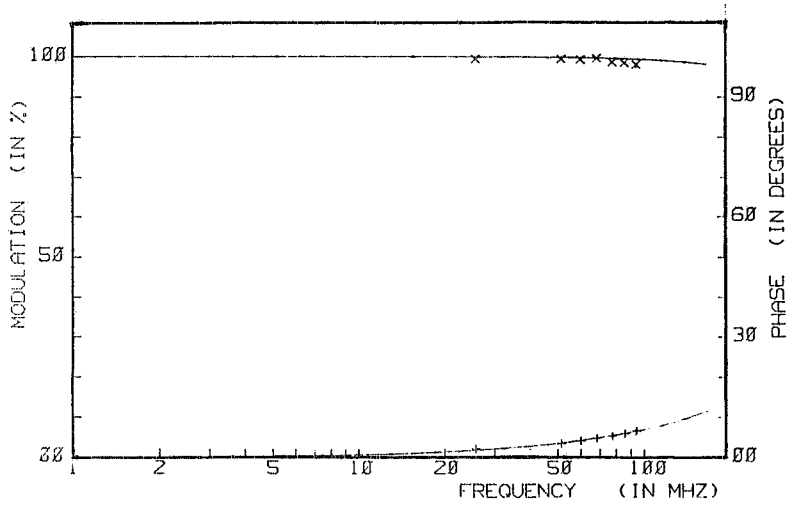

FIG. 7 - Multifrequency phase (+) and modulation (x) data for bis-ANS in water at 20°C. Solid lines correspond to the best fit using a single exponential component $\tau_o = 196 \pm 10$ psec.

The excitation wavelength was 370 nm (8 nm slits) and the emission was observed using a cut-off filter ($\lambda_{EM} > 420$). The least-squares analysis of the data show a single component of 196 ± 10 psec. For bis-TNS in water an even shorter lifetime, 113 ± 5 psec, was measured. We should note that at the normal optical densities used (< 0.1 O. D.) the emission intensities of these compounds are comparable to the water Raman band intensity.

4. 2. - Tryptophan studies

4. 2. 1. - Rationale

An extensive study was performed on tryptophan solutions at two different pH values. Tryptophan is the most important natural occuring fluorescent amino-acid in proteins and its fluorescence properties have been largely characterized although controversies persist regarding the molecular origin of the emission properties. Our measurements confirm some of the findings which has been debated in the literature.

4. 2. 2. - Neutral pH; single exponential decay

Phase and modulation values of a tryptophan solution at pH = 6.9 in 55 mM sodium phosphate buffer at 20°C were measured. The excitation wavelength was 280nm

using 4 nm slits and the emission was analyzed using a Corning 0-52 filter (λ_{EM} > 350 nm). The results are presented in Fig. 8. The analysis using the

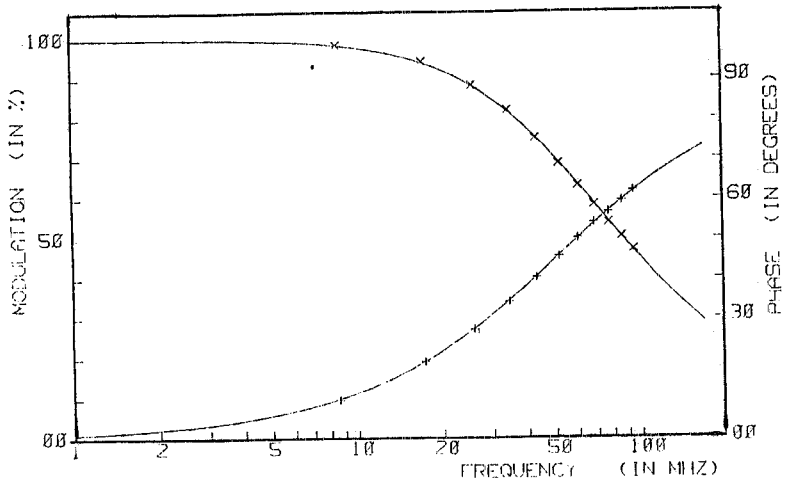

FIG. 8 - Multifrequency phase (+) and modulation (x) data for tryptophan at 20°C pH = 6.9. Solid lines correspond to the best fit using two exponential components τ_1 = 3.106 ± 0.013 nsec, τ_2 = 9.00 nsec and f_1 = 0.968 ± ± 0.005.

non-linear least-squares routine gives a double exponential decay with a very small amount of a long component, probably corresponding to a small fraction of the anion form present at high pH (Table III).

TABLE III - Results of heterogeneity analysis on tryptophan lifetimes. Two component analysis.

Condition	τ_1 (nsec)	τ_2 (nsec)	f_1	χ^2
pH 6.9 λ_{EM} > 350 nm	3.106 ± 0.013	9.000 (fixed)	0.968 ± 0.006	0.852
pH 9.25 λ_{EM} > 350 nm	3.193 ± 0.047	9.000 (fixed)	0.396 ± 0.099	6.093
pH 6.9 λ_{EM} = 313 nm	3.117 ± 0.275 3.106 (fixed)	0.742 ± 0.878 0.710 ± 0.218	0.896 ± 0.104 0.900 ± 0.016	16.927 4.181

4.2.3. - Alkaline pH; double exponential decay

Using the same experimental conditions described in the previous paragraph, phase and modulation values of a tryptophan solution at pH = 9.25 were measured and the results reported in Fig. 9. The solid lines correspond to the best fit obtained using two exponential components. The derived values for the lifetime and frac-

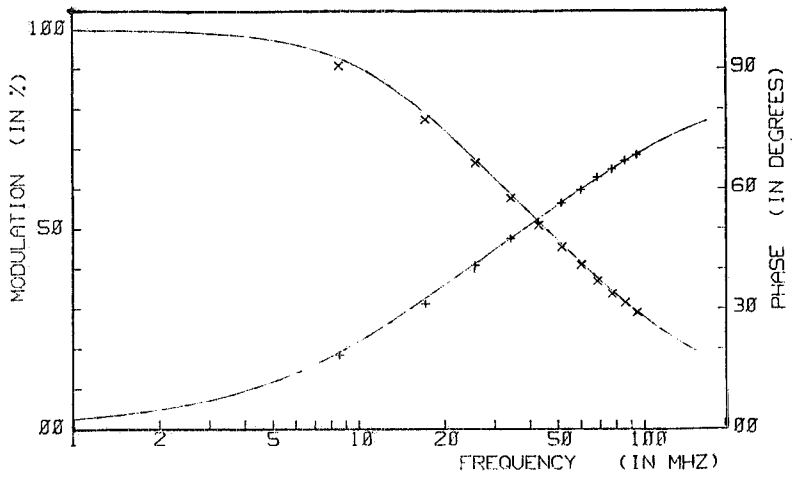

FIG. 9 - Multifrequency phase (+) and modulation (x) data for tryptophan at 20°C pH = 9.25. Solid lines correspond to the best fit using two exponential components $\tau_1 = 3.193 \pm 0.026$ nsec, $\tau_2 = 9.00$ nsec and $f_1 = 0.396 \pm 0.005$.

tional contribution of each component are also reported in Table III. The component lifetimes are in good agreement with the values expected for a mixture of the zwitterionic and anionic form of tryptophan. This experiment shows also the resolving power of the instrumentation for a two component system.

4. 2. 4. - Short wavelength component

The decay of the tryptophan emission was measured at emission wavelength of 313 nm using an interference filter (bandwidth 4 nm). The aim of this experiment was to test for the existence of a fast component at short wavelength as reported by Rayner and Szabo[22]. The result of the two component analysis using the non-linear least-squares routine is presented in Table III. The results are in close agreement with the values reported by Rayner and Szabo; also the error on the short component is markedly reduced when the value of the long component is fixed in the analysis.

4. 3. - Energy transfer studies

4. 3. 1. - Rationale

Tyrosine fluorescence is difficult to observe in proteins containing tryptophan for several reasons including the high efficiency of energy transfer from tyrosine to tryptophan[23]. The efficiency of energy transfer depends on many parameters such as the distance between acceptor and donor, the overlap between donor emission and acceptor excitation spectrum, the nature of the medium between donor and acceptor and geometrical orientational factors. From the acceptor point of view the excitation

due to the transfer of energy from the donor is delayed with respect to a direct excitation. The extent of delay depends on the transfer rate and on the lifetime of the donor. In the experiments described in this section we directly measured this delay. A comprehensive discussion of the observed transfer process is in preparation. Here we present some results of energy transfer experiments from tyrosine to tryptophan in bovine serum albumin (BSA) and between tyrosine-tryptophan and bis-ANS adsorbed to BSA.

4. 3. 2. - Tyrosine to tryptophan transfer

The results of a multifrequency study of the tryptophan lifetime of BSA are reported in Table IV. Three different excitation wavelengths were used, 250 nm, 270 nm and 300 nm each with a 4 nm bandwidth. The emission was observed through a Corning 0-25 filter ($\lambda_{EM} > 350$ nm) to select only the tryptophan emission. The major feature of the transfer process is revealed by the lengthening of the phase lifetime at high

TABLE IV - Tyrosine-tryptophan energy transfer in BSA. Phase and modulation lifetimes in nanoseconds as a function of excitation wavelength and modulation frequency.

MHz	λ_{EXC} 250 nm		λ_{EXC} 270 nm		λ_{EXC} 300 nm	
	τ^P	τ^M	τ^P	τ^M	τ^P	τ^M
8.568	6.608	7.154	6.129	7.252	6.203	7.468
17.137	6.372	6.728	6.373	6.888	5.889	6.889
25.705	6.516	6.606	6.226	6.842	5.513	6.583
34.274	6.417	6.529	6.190	6.772	5.297	6.387
42.842	6.483	6.472	6.425	6.507	4.994	6.250
51.411	6.450	6.502	6.708	6.663	4.730	6.104
59.979	6.669	6.445	6.696	6.567	4.442	6.065
68.548	6.881	6.371	6.874	6.580	4.483	6.002
77.116	6.873	6.262	6.890	6.503	4.160	5.918
85.685	6.721	6.322	7.465	6.391	3.917	5.820
94.253	6.676	6.496	7.558	6.417	3.617	5.700

modulation frequencies and by the inversion of the τ^P and τ^M values. To qualitatively interpret these results consider first the data for 300 nm excitation. At all modulation frequencies $\tau^P < \tau^M$ suggesting a double exponential decay of the emission. The result of the least-square analysis in terms of two exponential components is shown in Table V. At 270 nm a maximum ratio of the tyrosine to tryptophan absorption is observed and consequently a lengthening of the phase lifetime is expected because of the transfer process. This phenomenon is clearly shown in Table IV at high frequency.

Also note that $\tau^P > \tau^M$, a condition achieved only in the presence of excited state reactions[24]. At 250 nm a lower ratio of tyrosine to tryptophan absorption exists and the lesser extent of energy transfer is shown by the decrease of τ^P at high frequency when compared with 270 nm excitation. A series of measurements were performed at a fixed modulation frequency (94.253 MHz) and τ^P was measured as a function of the excitation wavelength. The data obtained are reported in Fig. 10a. Qualitatively, we can assign a 'transfer excitation spectrum' to a species with a maximum absorption at 270 nm. A more detailed study is necessary to reveal the spectrum of the donor. We emphasize that we discuss here only some qualitative aspects of the transfer process and that a more detailed study is in progress.

TABLE V - Two component heterogeneity analysis for BSA excited at 300 nm.

τ_1	= 6.828 ± 0.165 nsec
τ_2	= 1.444 ± 0.196 nsec
f_1	= 0.884 ± 0.017
χ^2	= 6.219

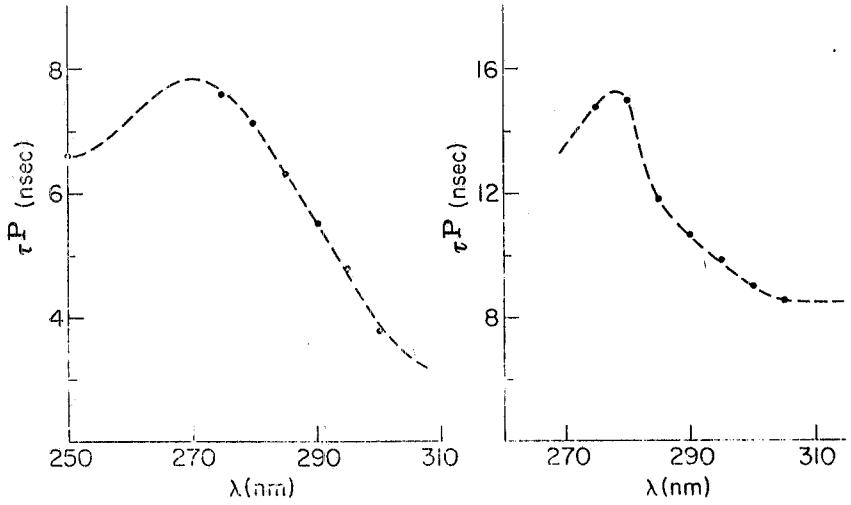

FIG. 10 - a) Phase lifetime as a function of the excitation wavelength for BSA. The emission is collected using a Corning 0-52 filter ($\lambda_{EM} > 350$ nm). b) Phase lifetime as a function of excitation wavelength for bis-ANS: BSA adducts. The bis-ANS emission is collected using a cut-off filter ($\lambda_{EM} > 420$ nm).

4. 3. 3. - BSA to bis-ANS transfer

The same features of the transfer process are displayed in the experiments performed on the bis-ANS: BSA adducts where tyrosines and tryptophans act as donors and bis-ANS is the acceptor. In Table VI we report the multifrequency lifetime study for two excitation wavelengths using 4 nm slits. The bis-ANS emission is observed through a cut-off filter ($\lambda_{EM} > 450$ nm). Excitation in the 300 nm region causes a

TABLE VI - BSA energy transfer to bis-ANS. Phase and modulation lifetimes in nanoseconds as a function of excitation wavelength and modulation frequency; $\lambda_{EM} > 470$ nm.

MHz	λ_{EXC} 275 nm		λ_{EXC} 370 nm	
	τ^P	τ^M	τ^P	τ^M
8.568	10.923	12.100	10.146	11.973
17.137	10.549	11.679	9.936	11.275
25.705	10.462	11.405	9.353	10.927
34.274	10.641	11.075	9.294	10.690
42.842	10.974	10.980	9.122	10.278
51.411	11.010	11.009	8.777	10.357
59.979	11.818	10.825	8.870	10.090
68.548	12.419	10.900	8.822	10.240
77.116	12.905	10.825	8.319	10.156
85.685	14.969	10.729	8.695	9.781
94.253	12.772	10.939	8.853	10.240

'transfer excitation spectrum' can be roughly defined (Fig. 10b). These experiments illustrate the possibility of measuring energy transfer in proteins between different donor-acceptor pairs.

4.4. - Color center studies

Preliminary measurements have been performed on lifetimes of color centers excited in the various absorption bands. The limited sensitivity of the detector in the infrared has prevented up to now the measurements of emission in crystals other than NaF. The luminescence intensity of color centers is very high at the peak of the main absorption bands but drops more than one order of magnitude for the excitation into the weaker bands due to transitions towards the higher excited states.

The measured lifetimes (see Table VII) are in good agreement with the values known from the literature for F and F_2 centers[30, 31].

TABLE VII - Lifetimes (in ns) of color centers in NaF at LNT.

Center	τ (this meas.)	τ other	ref.
F	51 ± 2	$\simeq 53$	(30)
F_2	10.2 ± 0.2	12.3 ± 1	(31)
F_3^+	8.55 ± 0.05	--	

Lifetime dependence on the excitation wavelength at LNT was studied for the F_2 center. As reported in Fig. 11 the lifetime remains constant over the main absorption band (480-510 nm) and also with excitation in the second band (341 nm).

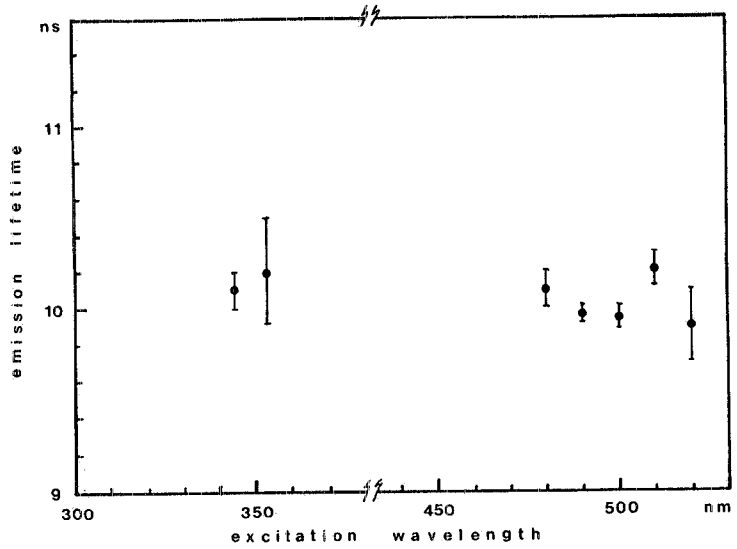

FIG. 11 - Emission lifetima of the first two excited states of the M centers in NaF as a function of the exciting photon wavelength at LNT. The centers were obtained by irradiating the NaF sample with γ-rays. The maximum of the absorption band for the M_1 state is at 498 mm and for the M_2 state is at 341 nm.

Independent measurements (in other color centers) indicate a phonon relaxation time of $\simeq 10$ ps[32]. Therefore the lengthening of the lifetime of emissions excited at higher energies is on the border of being accessible to our technique.

ACKNOWLEDGMENTS

We acknowledge the assistance of the staff of the Divisione Macchine of the Laboratori Nazionali INFN of Frascati, and the help of the RF group, in particular of Ing. R. Boni. One of us (N. R.) is also grateful to the staff of the Istituto di Biofisica of CNR, Pisa.

Financial support for this research was provided in part by NSF project 82-FR--47, by the CNR grant no. 82-00698-02 and by the CNR-INFN joint program PULS. One of the authors (E. G.) gratefully thanks the Istituto di Struttura della Materia of CNR for the financial support during the six months period of activity at PULS.

APPENDIX - Quantitative comparison between single-photon-counting and phase techniques using synchrotron radiation.

A. - LIGHT INTENSITY

In normal SPC operation a maximum of one photon is detected for each incident light pulse. This limitation is of no consequence if the average number of emitted photons per second is less than the average number of pulses per second since in that case all emitted photons are collected. In phase fluorometry all photons are collected irrespective of the average number of emitted photons per second.

In our experiments using synchrotron radiation at ADONE we have measured an average photocurrent of about 10^{-6} A in a typical fluorescence experiment which translates to about 10^6 photons detected per second given the gain of photomultiplier (Hamamatsu R928 at 900 volts). This signal is quite high for single photon counting experiments and in that mode some of the photons could be lost. However, it is fair to say that if such a signal intensity is available, SPC experiments are very easy to perform. Measurements have also been carried out with an intensity condition corresponding to 1000 to 10000 photons per seconds using an integration time of 8 seconds and reliable values of phase and modulation have been obtained. We believe that a lower limit for the light level for the present phase instrumentation is about 100 to 1000 photons per second. This figure provides a quantitative evaluation of the sensitivity of a phase fluorometer.

In conclusion one would say that phase methods or SPC methods are comparable as regards sensitivity. If the signal intensity is low then both methods use all the photons and are equivalent: if the signal intensity is high, although phase methods make better use of all the photons, the sensitivity of single photon counting is sufficient for all practical purposes.

B. - DECONVOLUTION FOR THE FINITE PULSE WIDTH

Using phase methods there is no deconvolution for the finite light pulse width and for the electronic response of the detection system because phase delays and modulation ratios are measured relative to the incident radiation. The problem of deconvolving for the finite pulse width of the system, which is a major problem of pulse methods[25, 26], is greatly simplified when synchrotron radiation is employed because the shape of the pulse is known a priori and is invariant from pulse to pulse. However, for the measurements of lifetime of the order of one nanosecond or less, the deconvolution can be important even using synchrotron radiation because the decay time can be within

the pulse width (depending on the particular synchrotron radiation source employed). With respect to the deconvolution problem, phase fluorometry offers a distinct advantage.

C. - DIFFERENTIAL MEASUREMENTS

Phase methods are intrinsically differential because the phase delay is measured with respect to some reference phase. The reference can be conveniently chosen and usually, for an absolute lifetime measurement, the phase delay is measured with respect to a scattering sample which is assumed to have the phase of the incident radiation. Also the modulation ratio of the emission is measured with respect to the modulation ratio of the scattering sample. Instead of the scattering sample a different reference can be chosen. For example, for the measurement of the decay of the emission anisotropy,the phase delay of the light emitted with parallel polarization (with respect to the excitation) is measured with respect to the phase delay of the light emitted with perpendicular polarization. Following the theory of differential phase fluorometry[5] this measurement, when carried out as a function of the frequency, is equivalent to the measurement of the decay of the emission anisotropy using pulse methods. The differential method has the same accuracy as the absolute lifetime measurement, because only two phase measurements are necessary in each case. Another interesting application of differential measurements is the detection of the delay of the emission of the blue and red part of the spectrum of certain molecules[4].

The direct differential method is not available for SPC. A differential operation can be performed, as shown recently[27], by deconvolution of the pulse response by an appropriate function. For example if the pulse response in the blue part of the spectrum is known for a given molecule, then the difference between red and blue emission can be obtained by deconvolution of the red response using the blue response. The differential deconvolution method is the equivalent of the differential phase (and modulation ratio) measurement. Needless to say, a computer is always necessary in order to obtain the desired deconvolution from the pulse response. For differential phase measurements the desired phase difference is directly available.

D. - HOW TO THINK OF PHASE METHODS IN TERMS OF SINGLE PHOTON COUNTING

A more quantitative comparison between phase fluorometry and single photon counting decay measurements can be made if we think to perform measurements of phase delay and modulation ratios using single photon counting techniques as sug-

gested below.

Suppose we fix the modulation frequency at 100 MHz. Consider now a period at that frequency and suppose we want to measure the phase and modulation of the fluorescence with respect to a reference. Divide the period in 360 bins of one degree each and count the photons in each bin for a fixed amount of time. Once the photons have been collected reconstruction of the emitted sine wave and determination of the phase delay and modulation ratio of the fluorescence with respect to the reference is straightforward. Instead of 360 bins we can equally well use 4 bins of 90 degree each. Furthermore instead of measuring phase delay and modulation ratio at 100 MHz we can continue to use the cross-correlation principle and perform the measurement at 24 Hz. In this case the cross-correlation product at the photomultiplier can be seen as a gating of the photomultiplier with a square wave that can be shifted by 90 for each bin. Evidently, using this method we lose one half of the photons because the photomultiplier is turned on for only half a period. The procedure suggested above is exactly what is done in phase fluorometry, however instead of using photon counting we use analog detection. Because the signal noise is very low in the bandwidth of our experiment (0.1 Hz at 100 MHz), the two methods, bin collection and analog detection are completely equivalent.

E. - CONCLUDING REMARKS

There may be some cases in which the time domain cannot be substituted by the frequency domain. In fact the frequency domain measurement requires a repetitive source. Such a source is not always available. The comparison here is made only for high repetition rate frequency sources. This requirement restricts us to synchrotron radiation, mode-locked lasers and modulation of CW sources. Furthermore, we are primarly interested in very fast decays, on the order of few nanoseconds to picoseconds. In this particular time range, the measurement in the frequency domain appears to be equivalent or superior to the direct time recording. This superiority is due to the higher sensitivity, to the fact that no deconvolution for the finite time response of the system is needed, to the possibility of performing direct differential measurements and to the high intrinsic accuracy of phase delay and modulation ratio measurements.

Finally, we note that phase methods provide an absolute measurement of the lifetime. The calibration of the time scale, an operation always necessary in the time domain, is not performed in the frequency domain because only frequencies, known with very high precision, are measured.

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