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THE FLUORESCENCE DECAY OF REDUCED NICOTINAMIDES IN AQUEOUS SOLUTION AFTER EXCITATION WITH A UV–MODE LOCKED Ar ION LASER

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Abstract—The fluorescence decay kinetics of the reduced nicotinamides NMNH, NADH and NADPH in aqueous solution were investigated using an Ar ion laser, mode locked in the UV, as source of excitation and single photon counting electronics in the detection system allowing for a time resolution in the picosecond range. Analysis of the experimental fluorescence decay showed that the dinucleotides did not follow a single exponential decay law. Good fitting was accomplished with a sum of two exponentials. The mononucleotide fluorescence decay was a single exponential for at least 95% of its amplitude.

The heterogeneity in lifetimes of the fluorescence of the dinucleotides was interpreted in terms of an exciplex mechanism.

INTRODUCTION

The fluorescence decay kinetics of the reduced nicotinamide nucleotides NMNH (reduced β-nicotinamide mononucleotide), NADH (reduced β-nicotinamide adenine dinucleotide) and NADPH (reduced β-nicotinamide adenine dinucleotide phosphate) have received considerable attention in the past decade (Scott *et al.*, 1970; Schuyler *et al.*, 1972; Brochon *et al.*, 1976; Gafni and Brand, 1976). In aqueous solution the quantum yield of the fluorescence is about 0.02 (Scott *et al.*, 1970) and the lifetimes are in the subnanosecond regime (Scott *et al.*, 1970; Schuyler *et al.*, 1972; Brochon *et al.*, 1976; Gafni and Brand, 1976). Binding to dehydrogenases or dissolving in glycerol results in distinct multi-exponential kinetics (Brochon *et al.*, 1976; Gafni and Brand, 1976). The mechanism giving rise to deviation from monoexponential behaviour has not been firmly established. The presence of different emitting species has been assumed to account for the observed heterogeneity (Brochon *et al.*, 1976). On the other hand the results were interpreted by the supposition of a still unspecified reversible excited state reaction (Gafni and Brand, 1976). The lifetimes observed in aqueous solution were assumed to be exclusively or nearly monoexponential (Schuyler *et al.*, 1972; Brochon *et al.*, 1976; Gafni and Brand, 1976).

In this communication we report the results of subnanosecond fluorescence decay experiments conducted with NMNH, NADH and NADPH in aqueous buffer. For this purpose the nucleotides were excited with 100 ps light pulses from a mode locked UV-line of an Ar ion laser. Using these short light pulses the time resolution of the pulse fluorometry system is improved by at least a factor of five as compared to systems employing discharge lamps with relatively long pulse duration (Schuyler *et al.*, 1972; Brochon *et al.*, 1976; Gafni and Brand 1976). The advantage is that a more accurate analysis of the time dependence of the fluorescence can be performed at short time intervals after the pulse. As a consequence the improved accuracy may provide a helpful means in elucidating the mechanism of deactivation of the singlet excited state of the reduced nicotinamides.

MATERIALS AND METHODS

NMNH (lot no. 85C-7120), NADH (lot no. 98C-72301) and NADPH (lot no. 89C-7410) were obtained from Sigma Chemical Company. Solutions were freshly prepared in 0.05 *M* sodium phosphate buffer (pH 7.0) made from glass distilled water. The concentration was adjusted such that the optical density at 351 nm was 0.20 per cm light path. 1,1,4,4-Tetraphenyl-1,3-butadiene (TPB) was obtained from Fluka A.G. and 1,4-diphenyl-1,3-butadiene (DPB) was purchased from Eastman Kodak. The compounds were dissolved in fluorescent grade cyclohexane (Merck) and the solutions were degassed on a vacuum line.

Absorption spectra were recorded on a Cary 14 spectrophotometer and fluorescence spectra were obtained with an Aminco SPF-500 spectrofluorimeter. The fluorescence lifetimes were measured with the following set-up (*cf.* also Visser and Van Hoek, 1979). The excitation source was a Coherent Radiation Ar ion laser, model CR18UV. Mode locking was applied by a brewster cut acoustooptic prism that was driven by a Harris mode-locker drive unit, synchronized by a Marconi TF2173 digital synchronizer (38.070 MHz). The 76.140 MHz optical pulses were monitored using a fast photodiode (Telefunken BPW28). The pulses had a full width at half maximum (FWHM) of about 100 ps when corrected for detector risetime. Various UV-lines can be mode locked (333.6, 351.1 and 363.5 nm) by careful adjustment of the mode locking prism. For the present measurements the line at 351.1 nm was employed.

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The laser output at 351.1 nm was 500 mW continuous wave and 100 mW averaged mode locked power. With a beam splitter–mirror arrangement about 7 mW averaged mode locked power was deflected towards the thermostatted sample compartment. At the input gate a filter holder was mounted to accommodate neutral density filters (Balzers). Fluorescence light, perpendicular to the exciting beam, passed through a Schott KV418 cut-off filter and through a polarizer rotated at 54.7° with respect to the vertical position to minimize the effect of rotational motion on the lifetimes (Spencer and Weber, 1970). The emission light was then focussed on the entrance slit of two 0.25 m Jarrell Ash monochromators in tandem with the wavelength set at 450 nm, bandwidth 10 nm. The light output of the monochromator was focussed on the photocathode of the photomultiplier for better time resolution. The photomultiplier was a Philips XP2020 in a thermoelectric cooled housing (Products for Research Inc.). The response of the detection system was measured by replacing the standard 1 cm cuvette in the sample holder by a 45° mirror. The monochromator was set at the exciting wavelength and the laser beam was attenuated with neutral density filters down to submicrowatt level. Scattering from freshly prepared glycogen solutions appeared to lengthen the pulse somewhat and was therefore not used to record the impulse response function of the system. The detection electronics were NIM-modules from the ORTEC 9200 series and a Laben 8001 multichannel analyzer. Using a fast amplifier and a constant fraction discriminator the time to pulse height converter was started by photon responses. The stop pulses were delivered by a 100 MHz discriminator shaping the 38.070 MHz sinusoidal reference signal of the modelocker driver. By attenuating the excitation beam with neutral density filters, the start rate of the time to amplitude converter was reduced to about 15 kHz. Data were collected during a single or multiple period of 100 s, controlled by the timer of the multichannel analyzer. The background from a buffer solution was collected during a same period of measuring time and was subtracted from the decay data. The time scale was calibrated using the Ortec 462 time calibrator. All measurements from the multichannel analyzer were recorded on a digital cassette tape recorder and the cassettes were read into a DEC-10 computer system for data analysis.

Deconvolution procedures were applied in order to account for the finite duration of the exciting pulse in the analysis of the decay. We used the nonlinear least squares method for deconvolution of fluorescence decay (Grinvald and Steinberg, 1974) assuming a single or double exponential decay law:

$$\sum_{j=1}^{n} \alpha_j \, e^{-t/\tau_j},$$

with $n = 1$ or $n = 2$, respectively. The parameters (α_j, τ_j) were adjusted, until the criterion of best fit was attained after a search for the minimum value of ϕ:

$$\phi = 1/N_0 \sum_{i=1}^{N} \{F(i) - F_c(i)\}^2/F(i),$$

$F(i)$ denotes the experimental fluorescence in channel i, $F_c(i)$ is the computed fluorescence response, N is the number of channels used and N_0 is a normalization factor defined as

$$\frac{1}{N} \sum_{i=1}^{N} 1/F(i).$$

For visual comparison plots of the deviation function and of the correlation functions of the residuals are generated. Details of the analyzing procedure and of the graphical presentation are given by Grinvald and Steinberg (1974).

RESULTS

Fluorescence lifetimes were determined with diluted solutions of DPB and TPB in cyclohexane in order to check the performance of the system and the method of analysis. DPB was chosen since it is characterized by a short single lifetime (Lewis *et al.*, 1973). TPB was selected as reference compound because its absorption and emission have a spectral distribution comparable to that of the nucleotides and the lifetime is longer (Brochon *et al.*, 1976). The results obtained with these standards are presented in Figs 1A and B. Fitting of the experimental data was also accomplished by convolving the instrumental response with a sum of two exponentials. In neither case did we find improvements in the fits. The ϕ-values of a double exponential fit are, respectively, 0.003 for DPB and 0.006 for TPB (*cf.* the values for a single exponential fit as given in Fig. 1). The shape of the autocorrelation function of the residuals was not changed in both cases. The exciting pulses (FWHM 100 ps) are broadened considerably by the detection system resulting in a FWHM of about 700 ps. This broadening is mainly due to transit time variations in the photomultiplier. With the photomultiplier used we were not able to detect a shift in response time as was reported by others (Easter *et al.*, 1976; Wahl *et al.*, 1974). Only the shape of the pulse as observed by the detection system might show minor variations (*cf.* below). The fact that the system is able to measure single lifetimes of fluorescent standards is essential for the reliability of the data analysis of the reduced nicotinamides.

Examples of decay profiles of the reduced nicotinamides NMNH and NADPH at 2° are given in Fig. 2. The experimental curves were convoluted with single and double exponentials. The relevant parameters are collected in Table 1, in which the results at other temperatures are also incorporated. Inspection of Fig. 2 and Table 1 leads to the following conclusions. Although there is a minor long component present in the mononucleotide, it only contributes for less than 5% of the total decay. The value of ϕ hardly improves as a result of a double exponential fit. Therefore the decay of NMNH in aqueous solution can be considered for more than 95% to be exponential. The lifetime is around 0.30 ns and is unaffected in the temperature range of 2–20°C. The fluorescence of the dinucleotides NADH and NADPH does not obey a single exponential decay law. This can be judged from the graphical representation of the fit with a single exponential and from the relatively high value for ϕ. Much better results were obtained by fitting the experimental data with a double exponential function. Note the distinctly lower ϕ-values in these cases. Both NADH and NADPH can be characterized with similar parameters, namely a predominant component with a time constant in the range of 0.25–0.30 ns and a component with a longer lifetime of around 0.70 ns at 20°C (0.80 ns at 2°C) and with a smaller amplitude.

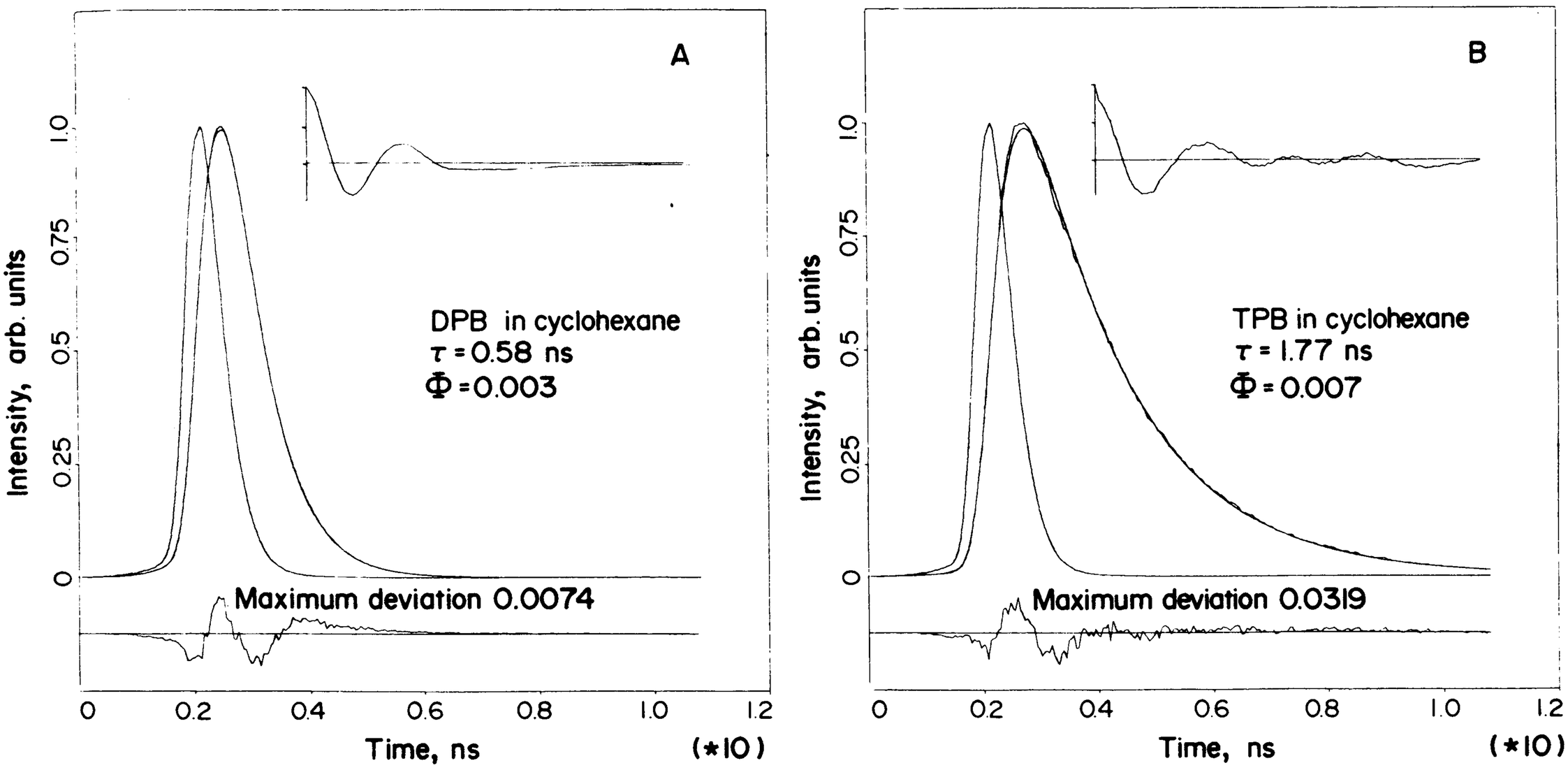

Figure 1. A: Laser pulse profile, experimental fluorescence impulse response of DPB in cyclohexane and computed decay curve assuming one single lifetime. All curves are normalized to the peak values (usually > 50000 counts). Parameter values are indicated in the figure. B: The same as in A, but now for TPB in cyclohexane. For details of data presentation see text.

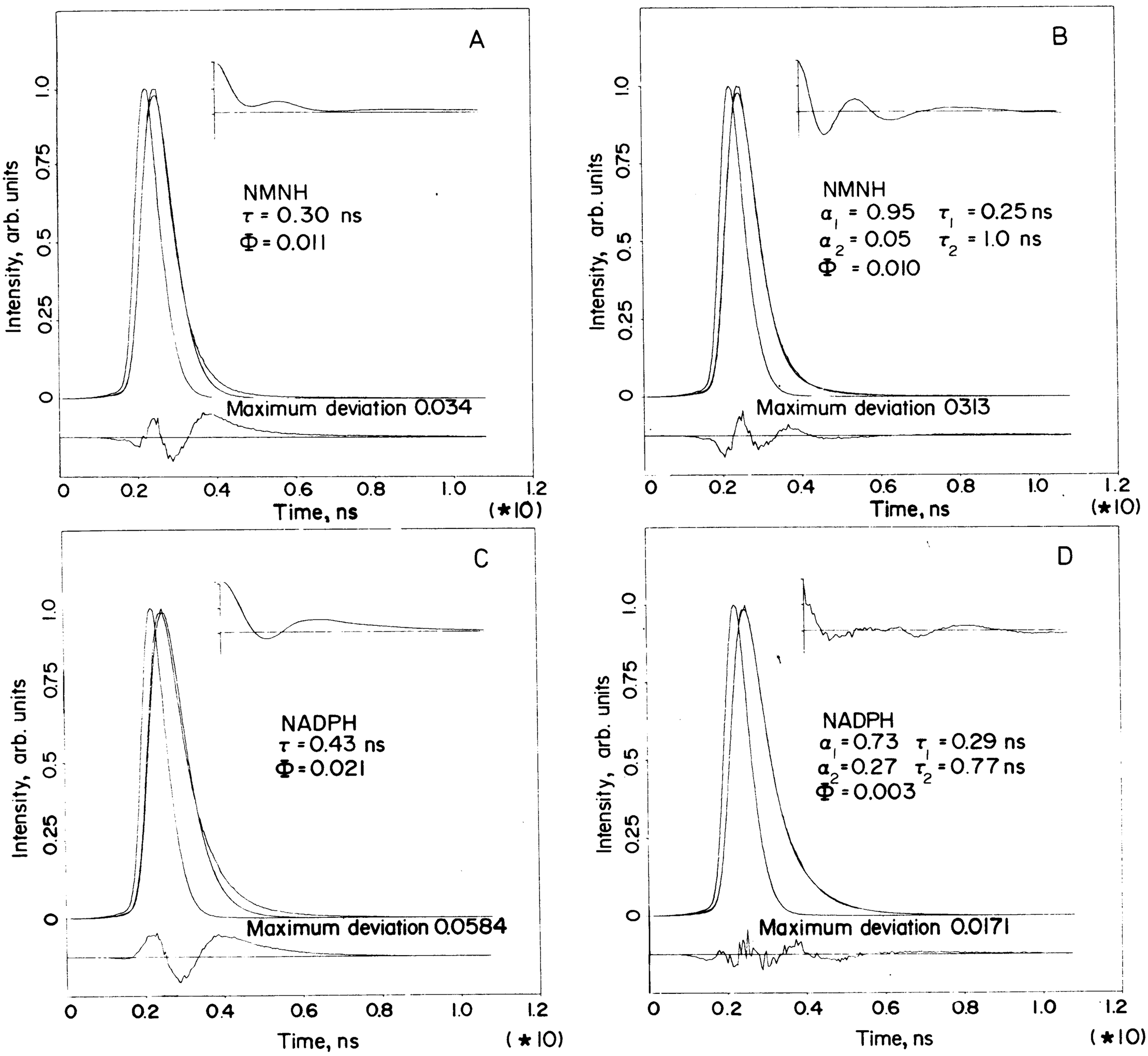

Figure 2. Fluorescence decay characteristics of NMNH and NADPH in 0.05 *M* sodium phosphate pH 7.0 at 2°. A and B: NMNH, single and double exponential fits. C and D: NADPH, single and double exponential fits. Parameter values are indicated in the figures. Further details are mentioned in the legend of Fig. 1 and in the text.

Table 1. Fluorescence decay parameters of reduced nicotinamides in aqueous solution*

Sample	T (°C)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$† (ns)	Φ $\times 10^4$
NMNH	20		0.29				58
		0.99	0.28	0.01	1.7 ± 0.3	0.36	50
NADH	20		0.40				118
		0.82	0.25	0.18	0.69	0.42	27
NADPH	20		0.39				74
		0.86	0.31	0.14	0.70	0.41	42
NMNH	11		0.29				112
		0.98	0.27	0.02	1.4 ± 0.2	0.38	105
NADH	11		0.41				129
		0.78	0.28	0.22	0.75	0.48	62
NADPH	11		0.41				131
		0.83	0.30	0.17	0.81	0.48	80
NMNH	2		0.30				108
		0.95	0.25	0.05	1.0 ± 0.2	0.38	99
NADH	2		0.49				142
		0.71	0.29	0.29	0.81	0.57	57
NADPH	2		0.43				210
		0.73	0.29	0.27	0.77	0.53	29

*The SD of the given parameters was always less than the most significant digit, unless otherwise indicated.

†The average lifetime is defined as

$$\langle\tau\rangle = \sum_{i=1}^{2} \alpha_i \tau_i^2 \Big/ \sum_{i=1}^{2} \alpha_i \tau_i .$$

The average lifetime of NADH and NADPH (*cf.* Table 1) becomes longer upon decreasing the temperature due to lengthening of the second longer lifetime component.

Two additional experiments were performed. The instrumental response was calculated from the fluorescence decay of the DPB solution by the method described by Wahl *et al.*, (1974) and was used in the deconvolution procedure for obtaining the parameters of NMNH and NADH at 20°C. The resulting parameters remain unaltered (*cf.* Table 1), implying that the shape of the instrumental response function hardly varies between 350 and 450 nm. The other experiment, that was carried out, concerned the variation of the intensity of the laserbeam impinging on solutions of NMNH and NADH at 2°C. The intensity was increased by a factor of 100 and the flux of emission photons was attenuated with neutral density filters to desired count rate. Also in these experiments analysis of the results yielded nearly the same values as presented in Table 1, implying that the decay kinetics are not affected by a change in light intensity.

Another important point should be emphasized, namely, the fact that the average lifetimes of both dinucleotides are distinctly longer than that of the mononucleotide. The same applies for the relative quantum yields. The ratio of quantum yields at 2°C (Q_{NMNH}/Q_{NADH}) is 0.55 and the ratio of lifetimes ($\tau_{NMNH}/\langle\tau\rangle_{NADH}$) amounts 0.53. These results are in sharp contrast with the fluorescence data of FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide), where in the latter compound quenching of the flavin fluorescence by the adenosine is evident (Spencer and Weber, 1972). Therefore, the mechanism describing the excited state kinetics of the reduced nicotinamides should be accounted for differently. The normalized emission spectra of NMNH and NADH (Fig. 3) do not coincide completely, the NMNH spectrum appears to be red shifted about 3 nm. The fluorescence spectra of NADH and NADPH are identical.

DISCUSSION

Earlier results obtained with the dinucleotides indicated that NADH and NADPH in aqueous solution

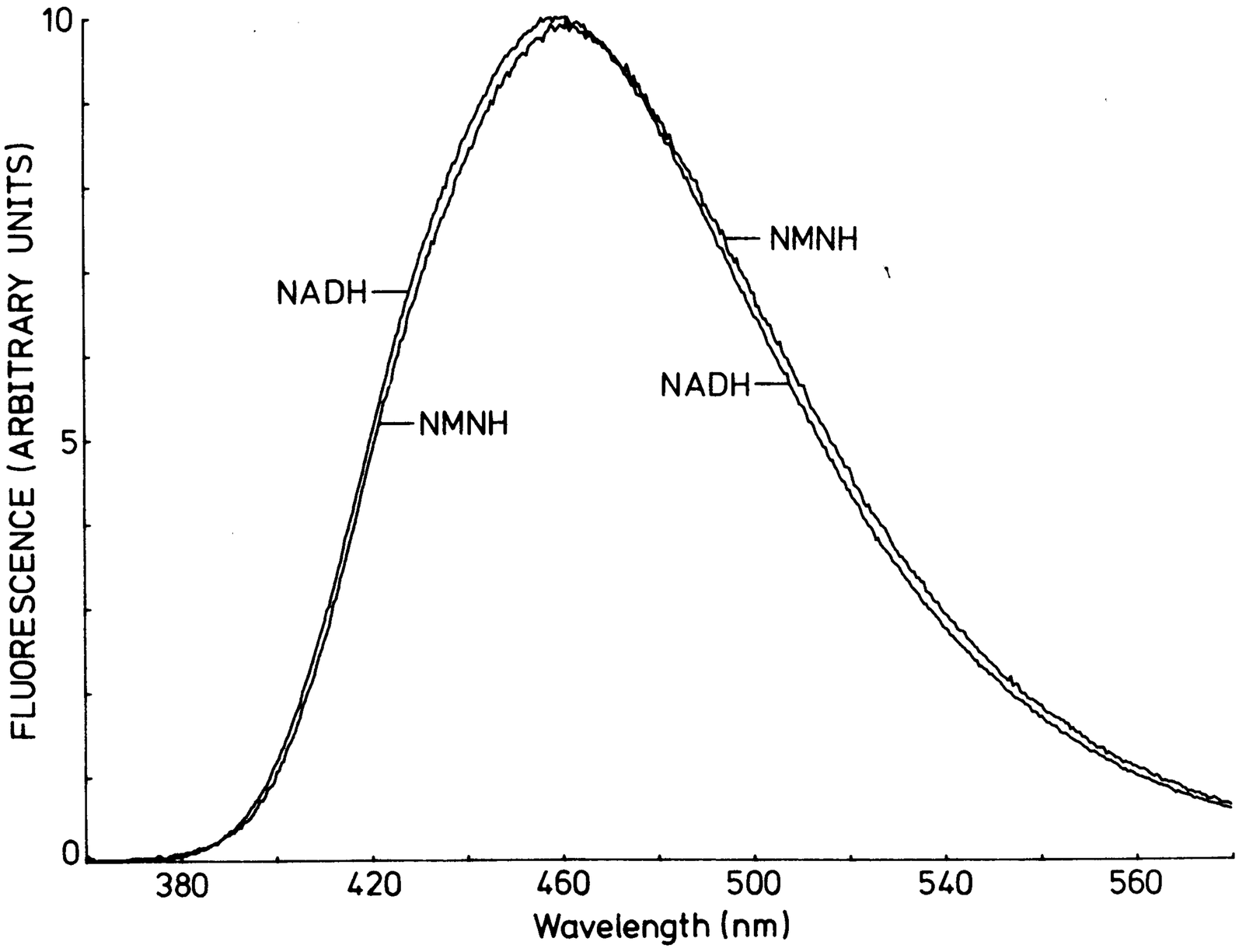

Figure 3. Peak normalized fluorescence spectra of NMNH and NADH in 0.05 *M* sodium phosphate pH 7.0 at 2°C. Excitation wavelength: 351 nm, band widths in excitation and emission 5 nm.

can be characterized by a single fluorescence lifetime of about 0.40 ns at room temperature (Scott *et al.*, 1970; Schuyler *et al.*, 1972; Brochon *et al.*, 1976; Gafni and Brand, 1976). The present experiments revealed that the decay is not governed by a single exponential decay law. It was shown previously for NADPH that a slightly lower ϕ-value was obtained with a simulation using two lifetimes of 0.36 ns (weighting factor 0.62) and 0.83 ns (weighting factor 0.38) at 0°C (Brochon *et al.*, 1976). It should be pointed out that previous measurements were made with excitation pulses > 2 ns, i.e. much longer than the lifetimes. The results of Brochon *et al.* (1976) are in perfect agreement with the results presented in Table 1. The significant difference is that our biexponential fits are characterized by much lower ϕ-values and consequently should be considered as more reliable. In the current experiments the exciting system had a characteristic time in the same order as the lifetimes. According to our intuitive feeling it is not possible to detect subnanosecond heterogeneous decay in case of an exciting pulse, which has a FWHM of a few nanoseconds. Phase fluorimetry has this potential, but only when several modulation frequencies are employed. The phase fluorimetric expression for a sum of two exponentials is

$$\sum_{i=1}^{2} A_i \tau_i^2 \Big/ \sum_{i=1}^{2} A_i \tau_i,$$

with

$$A_i = \alpha_i/(1 + \omega^2 \tau_i^2)$$

When the decay parameters as given in Table 1 (e.g. NADH, 20°C) are substituted in this expression a phase lifetime (modulation frequency $\omega/2\pi = 28$ MHz) of 0.41 ns for NADH is obtained, in very good agreement with the experimental value of 0.40 ns (Scott *et al.*, 1970). The average lifetimes of Table 1 are also in good accord with the reported lifetimes from pulse fluorimetry (Schuyler *et al.*, 1972; Brochon *et al.*, 1976; Gafni and Brand, 1976).

Two possible explanations for the apparent heterogeneity in lifetimes have been put forward. The presence of different folded and unfolded configurations in dynamic equilibrium is an attractive assumption to explain the observations (Brochon *et al.*, 1976). The alternative explanation, namely, the supposition of a reversible excited state reaction, is equally plausible, since it also accounts for the heterogeneity of the fluorescence decay of NMNH in glycerol (Gafni and Brand, 1976). This excited state reaction might be related to photoionization, which is, according to a report of Robinson *et al.* (1977), a fairly common mechanism in aqueous solutions of molecules containing electron donating groups.

An increase of the excited state population by enhanced light intensity does not appear to affect the experimental rate parameters. Moreover, the fluorescence lifetime of the mononucleotide in neutral, aqueous solution is homogeneous. Since this lifetime also arises in the fluorescence decay of NADH and NADPH an alternative model is proposed. In the unfolded state the configuration of the dinucleotide mimics that of the mononucleotide explaining the short lifetime component. In the closed conformer, the fluorescence is not quenched, a situation occurring in FAD, but the stacked complex itself is fluorescent with an inherent lifetime of 0.70–0.80 ns. The exciplex (or charge transfer excited state complex) is also characterized by a slightly blue shifted emission spectrum relative to that of the mononucleotide. Speed and Selinger (1969) have derived the decay kinetics for formation of exciplexes. The fluorescence of the monomer depends on time as a sum of two exponential terms, whereas the exciplex emission decays as the difference between two exponential functions (Speed and Selinger, 1969). The rate constants depend on the excited state lifetimes and on the rate of interconversion of both molecular species. Since the emission spectra of mono- and dinucleotides overlap considerably (Fig. 3), the actual rate law is probably a linear combination of these exponential functions. Interconversion in the excited state in case of the dinucleotides is possible, but, if we consider the case of FAD (Spencer and Weber, 1972), the time constant for exciplex formation is in the order of 5 ns at 20°C and of 25 ns at 2°C, i.e. much longer than the excited state lifetimes of the reduced nicotinamides. Since an equilibrium between folded and unfolded dinucleotide conformations is present, both types of conformers can be excited and can fluoresce almost instantaneously, each with its own lifetime. At lower temperatures the longer lifetime component becomes more significant, implying that the ground state equilibrium is driven towards association.

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