Fluorescence Decay Times: Proteins, Coenzymes, and Other Compounds in Water

Abstract. *The fluorescence decay time (τ) was 2 to 5 nanoseconds for proteins and 4 to 5 nanoseconds for flavin, pyridine nucleotide, and vitamin B_6 coenzymes; τ varied widely in 48 compounds measured in water. Although reported values of τ for a few of the solutions studied were in excellent agreement, previously "calculated" lifetimes, in several instances, are apparently erroneous. Nonexponential decay was detectable with our "nanosecond-flash" apparatus, a modification of the first commercially aavilable unit for determination of τ.*

The fluorescence decay time τ is a significant parameter of fluorescence, but the direct measurement of this quantity has proved difficult since, for organic molecules, τ is of the order of 1 to 50 nsec (1 nsec $= 10^{-9}$ sec). Of the recorded values for τ for organic compounds, most have been obtained in a few physical laboratories concerned with investigating electronic spectra, and these were measured in nonaqueous solvents such as cyclohexane which favor high quantum yields and are suitable for studying highly structured spectra. Few decay times have been measured for compounds in water, especially those whose emission occurs in the ultraviolet; that is, at wavelengths below 400 mμ. There has been great interest in flourescence and other manifestations of excited electronic states in biological systems (*1*), not only because these states occur naturally (as in photosynthesis and firefly luminescence, for example) but also because luminescence techniques are valuable for studying the structure of biopolymers and their interactions with small molecules (*2*). It is therefore important to measure all basic fluorescence properties, such as τ, in aqueous solutions with equipment sensitive to ultraviolet radiation, since many biological materials, such as proteins, emit in this region. We now report τ values for 48 compounds in aqueous solutions including proteins and flavin and pyridine nucleotide coenzymes.

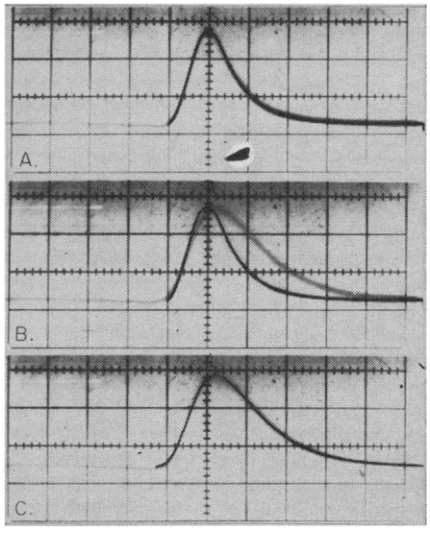

Fig. 1. Oscilloscope tracings showing steps in the determination of τ. In each frame, the sharper, darker curve is that produced by the curve simulator displayed with a baseline of 0.20 msec/cm; and the lighter curve is the signal observed with the phototube displayed at 20 nsec/cm. Each major division is 1 cm. (A) Scatter signal from N_2 flash lamp, and curve simulating that signal. (B) Simulated lamp pulse and observed fluorescence signal for acridone. (C) Observed fluorescence signal for acridone and curve simulator signal for lamp flash plus delay corresponding to τ of 15.4 nsec.

The process of fluorescence can normally be described by

$$I = I_0 e^{-kt}, \qquad (1)$$

a relation indicating that the fluorescence intensity, I_0, at the moment that excitation ceases, decays exponentially and is I at any given time t. The decay time τ is defined as the time when $I = I_0/e$, and it is therefore the reciprocal of the rate constant k. The decay time τ is also related to the quantum yield Q by the simple relation (*3*)

$$\tau = \tau_0 Q \qquad (2)$$

where τ_0 is the "natural lifetime" in the absence of radiationless transitions, in cases where fluorescence is partly quenched by simple collisional mechanisms.

Valuable information is obtained if the observed fluorescence does not conform to Eqs. 1 and 2. Nonexponential decay may indicate energy transfer between molecules (*4*), or that more than one emitting species is present. Equation 2 can be tested if τ can be mesured, because τ_0 can be calculated from the absorption spectrum (*5*) and Q can be measured. Deviations from that relation occur when quenching occurs owing to complex formation rather than collisions (*6*). Measurements of τ are therefore essential to the understanding of interactions leading to quenching.

Perhaps as a result of testing possible new laser materials, an instrument has become commercially available for determining fluorescence decay times (*7*). The principle of the apparatus has been described by Mackey *et al.* (*8*), and we have modified the instrument to improve the accuracy of measurements of the short decay times often found in aqueous solutions. Essential to the system is a nanosecond flash lamp operated at repetition rates up to 5 kc (we used 2 kc) by a thyratron-triggered pulsing circuit. Two lamps were used in our study. One contained N_2, which has intense emission but only above 297 mμ; the other was deuterium-filled, having less power but with most of its emission in the ultraviolet below 300 mμ. Light from the lamp is focused onto a sample through a primary filter, and emission passes through a secondary filter and is collected by lenses onto the photocathode of an RCA 1P28 photomultiplier tube. The signal is displayed on one channel of a dual gun oscilloscope (*9*). To determine fluorescence lifetimes, we first place a light-scattering suspension (Ludox, DuPont) in the sample cuvette and place identical filters in primary and secondary positions. The observed scatter signal has the general shape of the lamp pulse but is stretched out in

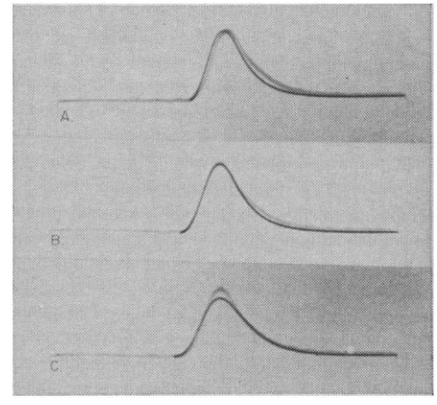

Fig. 2. Oscilloscope records showing nonexponential fluorescence decay of concentrated ($10^{-3}M$) acridine orange. Darker, sharper curves are those of the curve simulator. The grid reticule has been removed. (A) Simulated N_2 lamp pulse and observed fluorescence signal. (B) Simulator curve with computer set at 2.9 nsec, and observed fluorescence signal. The curves match at the beginning of the decay. (C) Simulator curve with computer set at 8.8 nsec, and observed fluorescence signal. The two curves in the tail match.

time owing to delay inherent in the electrical components. The other channel of the oscilloscope is connected to the instrument's analog computer to display a signal whose shape can be varied by the operator to match that of the scattered light signal (Fig. 1A). The scattering suspension is then replaced by the fluorescent solution, and the filter in the primary position is replaced by one suitable for the solution under study. The fluorescence signal is displayed which, because of the delay between absorption and emission of light, is always broader than the lamp pulse by an amount determined by the decay time (Fig. 1B). The operator determines the corresponding decay time by turning a potentiometer dial on the computer, thereby changing the shape of the simulated curve until it matches that of the observed fluorescence (Fig. 1C). During this operation, the computer's analog circuit is changed by introduction of an electrical delay which is an exact analog of the delay due to the time required for the fluorescence to rise and fall. The calibrated potentiometer is coupled to a dial which gives a direct digital read-out of the corresponding decay time. Details of the circuitry and lamps are available from the manufacturer.

Our modifications include a temperature-controlled chamber, an iris diaphragm to reduce the size of the scatter signal to that of the fluorescence, and the use of a scattering suspension to monitor the shape of the light pulse. We were able to dispense with the lamp-pulse monitoring unit supplied with the apparatus; namely, a fiber optic probe attached to the lamp housing and to a second photomultiplier. In our modification, the lamp pulse and fluorescence are seen with the same phototube and oscilloscope gain settings. Therefore, lamp and fluorescence signals undergo precisely the same amount of pulse stretching because of electronic delay. The precision of the measurements is adequate for all conceivable purposes; we estimate it to be $\pm$ 0.5 nsec, regardless of the decay time in the range of τ's encountered in our study. The accuracy of the values was checked by comparison with the most reliable reported values. The decay times (in nsec) obtained for quinine (19.0) and acridone (15.4) agree almost exactly with reported values (*10, 11*). Reported values for fluorescein (*10*), acridine orange (*12*), and proflavin (*13*) are also in excellent agreement. After our study was completed, we became aware of Konev's data on the lifetimes of several proteins (*14*). Although details of those measurements are not available to us, his values for trypsin, chymotrypsin, human serum albumin, lysozyme, and tryptophan are consistent with ours; but his value of 1.6 nsec for chymotrypsinogen is lower than ours. Except for these cases, direct measurements of the fluorescence decay times of water solutions of all the other compounds listed in Table 1 have not yet, to our knowledge, been recorded.

Table 1 has several features of particular interest. (i) Decay times of proteins vary considerably, but in most cases the decay time is as long or longer than that of free tryptophan even though it is known that the quantum yield of the tryptophan fluorescence of protein is often below that of free tryptophan (*15*). Equation 2 is thus insufficient to describe the emission process in proteins. The relatively long

Table 1. Fluorescence decay times (τ) for organic compounds in water at 23°C. Unless specifically noted, the solutions contained 0.01 *M* tris-Cl^-, *p*H 7.0, and solute concentrations were 1.0 mg/ml for proteins and 1×10^{-5} *M* for all other compounds. Filter numbers refer to Corning filters. Where the secondary filter position was open or contained only a glass plate, the observed signals were, with confidence, differentiated from scatter because (i) no signal was observed from a buffer blank, (ii) the sample signals were broader than the lamp pulse, and (iii) a glass plate in the exciting beam extinguished the signal. The D_2 lamp was used for ultraviolet fluorescence decay times. For visible fluorescence, τ was obtained with either D_2 or N_2 lamp, or both; substitution of one lamp for the other did not change the observed τ. The solutions were not de-aerated.

Compound	Primary, secondary filters	τ (nsec)
Ultraviolet fluorescence		
Albumin, bovine serum	Interference,* glass	4.6
Albumin, egg	Interference,* glass	4.5
Albumin, human serum	Interference,* glass	4.5
Apomyoglobin, horse heart	Interference,† glass	3.7
Apomyoglobin, sperm whale	Interference,† glass	3.0
α-Chymotrypsin	Interference,* glass	3.4
Chymotrypsinogen	Interference,† glass	2.9
γ-Globulin, human	None, glass	3.2
Glutamic dehydrogenase	Interference,* glass	4.1
Indole	Interference,* glass	2.7
Indoleacetic acid	7–54, 3–75	2.6
Lysozyme, hen	Interference,* glass	2.0
Pyridoxal, 10^{-4} *M*	7–37, 7–39	4.2
Resorcinol	Interference,* glass	1.7
Serotonin, 10^{-4} *M*	Interference,† glass	2.7
Skatole, saturated solution	Interference,* glass	6.4
Sulfanilic acid	7–54, 3–75	2.5
Trypsin	Interference,* glass	2.0
Tryptophan	7–54, glass	2.6
Tyrosine, 10^{-3} *M*	Interference,* none	2.6
Visible fluorescence		
Acridine orange	7–54, 3–75	2.0
Acridone, saturated solution	7–60, 3–73	15.4
1-Aminonaphthalene-4-sulfonic acid	7–54, 3–75	11.5
1-Aminonaphthalene-5-sulfonic acid	7–54, 3–73	5.5
1-Anilinonaphthalene-8-sulfonic acid, 10^{-3} *M*	7–54, 3–75	2.3
Anthranilic acid	7–54, 3–73	8.4
Coproporphyrin I, 2×10^{-6} *M*, 0.01 *M* HCl	7–54, 3–70	7.1
1-Dimethylaminonaphthalene-5-sulfonic acid	7–60, 3–73	13.8
Eosin Y, 0.1 *N* NaOH	7–60, 3–75	1.7
Flavin adenine dinucleotide	7–60, 3–73	4.9
Flavin mononucleotide (FMN)	7–60, 3–73	5.6
Fluorescein, 10^{-6} *M* in 0.1 *M* NaOH	7–54, 3–73	4.5
3-Hydroxyanthranilic acid	7–60, 3–75	10.9
6-Methoxyquinoline, 0.1 *N* H_2SO_4	7–54, 3–70	22.8
4-Methylumbelliferone	7–54, 3–70	5.6
NADH, 0.1 *N* $NaHCO_3$	7–60, 3–73	4.5
NADPH, 0.1 *N* $NaHCO_3$	7–60, 3–73	4.3
Nicotinamide mononucleotide, reduced, 0.1 *N* $NaHCO_3$	7–60, 3–73	3.8
Proflavin	7–60, 3–75	4.5
Protoporphyrin I, 0.01 *N* HCl	7–60, 3–73	7.2
γ-(1-Pyrene)-butyric acid, 0.1 *N* $NaHCO_3$	7–54, 3–75	93.1
Pyridoxamine-5-phosphate	7–54, 3–75	4.3
Quinine, 0.1 *N* H_2SO_4	7–54, 3–70	19.0
Quinacrine, 2×10^{-5} *M*	7–60, 3–73	4.0
Rhodamine 3GO	7–54, 3–73	3.9
Rhodamine 6GO	7–54, 3–73	5.8
Riboflavin	7–60, 3–73	4.2
Salicylic acid	7–54, 3–70	3.9

* 270-mμ interference filter from Optics Technology, Inc., Palo Alto, Calif. † 290-mμ interference filter from Optics Technology.

decay time of proteins may thus be due to tyrosine-tryptophan energy transfer or to noncollisional quenching due to intramolecular bonding or to both. However, a small deviation from exponential decay due to energy transfer in this lifetime range would not be detected by our system. (ii) The decay times of about 4 nsec for reduced nicotinamide-adenine dinucleotide coenzymes (NADH) are considerably higher than that of 0.3 nsec calculated by Weber (*16*) if Eq. 2 applied. On the other hand, his calculations on the lifetimes of the flavin coenzymes are more or less correct (*17*). From his calculation of τ for flavin-adenine dinucleotide (FAD) he postulated an equilibrium between fluorescent and nonfluorescent coenzyme forms in solution; and we therefore suggest that such an equilibrium also exists for the NAD coenzymes. Our postulate is supported by evidence from chemical relaxation data (*18*). (iii) The fluorescence lifetimes of several compounds are relatively long; for example, 6-methoxyquinoline (the parent compound of quinine), the porphyrins, anthranilic and 3-hydroxyanthranilic acids, and the naphthalene sulfonates. Protein complexes containing these compounds may therefore be useful in hydrodynamic studies on proteins by fluorescence polarization, in that such studies require long fluorescence decay times of the order of the protein rotational relaxation time (*19*). In our study, γ-(1-pyrene)-butyric acid had the longest lifetime observed, 93.1 nsec; the compound has already been used in studies of fluorescence polarization in proteins (*20*). (iv) Several directly determined values of τ (Table 1) conflict with those predicted by calculations based on certain assumptions. The case of NADH and NADPH has been mentioned; and the decay times, 2.6 nsec and 2.7 nsec, for tyrosine and indole are much shorter than predicted (*21*). These discrepancies emphasize the importance of direct τ measurements.

The apparatus also detects gross deviations from exponential decay (Fig. 2). The fluorescence signal from $10^{-3}M$ acridine orange could not be matched by the curve simulator. However, by matching the simulated curve first to the beginning of the decay of the fluorescence signal and then to the end (Fig. 2, B and C), apparent decay times of 2.9 and 8.8 nsec were read from the computer dial. Such nonexponential behavior of acridine orange fluorescence in concentrated solutions is due to the fluorescence of aggregates and intermolecular energy transfer (*12*).

Finally, we expect that the availability of convenient and rapid apparatus for decay time measurement will have considerable impact on studies of fluorescence, which have too long depended on assumed and calculated τ values.

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Drug-Induced Tolerance for Skin Allografts across the H-2 Barrier in Adult Mice

Abstract. *Mice treated with methylhydrazine derivatives for 3 to 4 weeks before the transplantation showed markedly prolonged survival times for allogeneic skin grafts differing at the H-2 histocompatibility locus. Presumably permanent tolerance was induced in about 20 to 30 percent of the mice when the drug treatment was combined with a single injection of additional donor antigen. The tolerance persists without further drug treatment and is specific for donor tissue.*

Immunologic tolerance for a variety of antigens has been achieved in adult animals with immunosuppressive chemicals, notably 6-mercaptopurine (*1*). Attempts to produce tolerance for allografts have been successful only with skin grafts involving identical H-2 histocompatibility genes (*2*, *3*) and with tumor grafts (*4*). It has also been shown that administration of massive doses of tissue antigen (*5*) and of tissue antigen combined with chemical immunosuppression (*6*) can make foreign grafts more acceptable. Efforts to induce pharmacologically tolerance for normal tissue grafts across the H-2 histocompatibility barrier in adult mice and other animals have not yet succeeded (*2*, *4*, *7*).

Long-term survival of canine and human renal allografts after cessation of immunosuppressive therapy may reflect a high degree of histocompatibility rather than tolerance; second transplants from the original donors are generally rejected (*8*).

Of the chemicals tested in my laboratory for prolongation of survival of first-set (*9–11*) and second-set allografts (*12*), methylhydrazine derivatives were the most effective. Because results were regularly better when administration of these drugs began 8 to 10 days before the transplantation (*9–11*), we explored the effects of longer pretreatment on transplantation immunity; I now report some preliminary results.

Two distinct strain combinations of donors with recipients (all fully grown mice), were used:

$A^{H-2^a} \leftarrow CBA^{H-2^k}$ and $C57Br^{H-2^k} \leftarrow A^{H-2^a}$

Both typified strong histocompatibility differences, with different alleles at the H-2 locus. With both combinations, grafts survived for 8 to 9 days in untreated mice; so the relatively less