Biochemical Fluorescence: Concepts

VOLUME 1

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PREFACE

In the 1960's, fluorescence spectroscopy became an important tool for the physical biochemist. Much of the reason for this popularity was due to the sensitivity and versatility of fluorescence methods as well as to the pioneering theoretical and practical work by investigators such as Förster and Weber. The last decade has seen an expanding interest in luminescence methods; indeed, we appear to be in a period where the literature on fluorescence is growing exponentially.

Luminescence phenomena involve ground and excited states, and the study of such phenomena may require different kinds of measurements, such as fluorescence yield, decay, polarization and spectral distribution. While the complexity of luminescence should not discourage investigators from using the technique, it is clear that this is really not a single method but a collection of methods, each of which has been developing as a result of the wide range of applicability of luminescence spectroscopy.

There is obviously no point in trying to assemble a "text" on fluorescence when the field is changing so rapidly, but there does seem to be a need to recapitulate some of the concepts and applications of fluorescence on which future advances will be based. Much understanding has already accumulated about the basis of fluorescence, polarization, energy transfer, quenching mechanisms, and spectral shifts, and has given rise to many valuable studies on specific biological systems. We now see further technical innovations brought about by the advent of the digital computer, the laser, and new electronic developments. A glance through the table of contents of this volume indicates that the area of decay kinetics has been opened up by technical advances, especially time-correlated single

photon methods and the associated computational theory. Other new techniques include circular polarization of luminescence, stopped-flow fluorescence, fluorescence-monitored chemical relaxation, and the evaluation of relative orientation by polarized excitation energy transfer.

Volume 2 will deal with some of the newer applications of fluorescence spectroscopy. New fluorescent probes and quenchers have helped to open up areas such as membranes, muscle and nerve components, and other subcellular organelles.

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CONTENTS OF VOLUME 2

CONTENTS

Cumulative Indexes will appear at the end of Volume 2.

Chapter 1

DECAY OF FLUORESCENCE ANISOTROPY

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I. INTRODUCTION

The fluorescence polarization of dye solutions was discovered by Weigert [1]. He found that polarization changes with the solvent viscosity. This was an indication that the Brownian motion is an important factor which affects the polarization.

The theory of the Brownian depolarization was given by F. Perrin [2,3] for rigid molecules of spherical and ellipsoidal shape. In In this theory, mathematical expressions are given which relate the degree of polarization to the lifetime of the dye, to the molecular volume (and to the axial ratio for ellipsoidal molecules), as well as to the temperature and the viscosity of the solvent [4,5,6]. One now prefers to use the emission anisotropy, introduced by Jablonsky [7], instead of the degree of polarization. We will use the emission anisotropy factor in this article.

The theory was first tested by Perrin [3] with chromophore solutions having different viscosities. These experiments were done with a steady excitation, and the quantities measured are what we shall call the static parameters. Perrin took the volume found by viscosity measurements [8] which enabled him to determine the lifetime of fluorescein which was in good agreement with the direct determination of Gaviola [9]. Perrin's theory, however, does not describe satisfactorily all the aspects of the experiments performed with aromatic molecules [10,11]. One of the reasons for this discrepancy is probably that these molecules are too small to obey the law of rotational Brownian motion.

On the basis of Perrin's theory, G. Weber [12,13] proposed a method for the determination of the morphological parameters of macromolecules in solution. In this method a relaxation time is obtained by measuring the static emission anisotropy at different temperatures, or in a series of solvents having different viscosities. This method has been applied to a variety of proteins, nucleic acids, and membranes labeled with fluorescent chromophores. At first sight,

one may think that macromolecules obey the laws of Brownian motion fairly well (see reviews of Chen et al. [14] and Dandliker et al. [15]). A critical analysis, however (Wahl [16], Wahl and Weber [17]), shows that, in many cases, the variation of the temperature or of the nature of the solvent may influence the anisotropy in a complicated way. Therefore a simple application of Perrin's formula is not justified and might even lead to a wrong interpretation of the experiments [18].

Perrin's theory predicts that the decay of the principal polarized fluorescence components $I_{\parallel}$ and $I_{\perp}$ are influenced by the Brownian motion. As Jablonski [7,19,20,21] has pointed out, the average lifetimes measured with a phase fluorometer are different for the whole fluorescence and for each of the polarized components. Coupling lifetime measurements in polarized light with measurements of static anisotropy should permit the determination of the fundamental anisotropy and the volume of a spherical chromophore. These data are obtained in a given solvent and at a given temperature. Experiments performed by Szymanowski [22], Kessel [23], and recently by Bauer [24] are in fair agreement with the prediction of Jablonski.

Spencer and Weber [25] have discussed in detail the use of the phase fluorometer to obtain the Brownian correlation times and the influence of the modulation frequency. Most of the present phase fluorometers work at one or two modulation frequencies [26]. The method might gain in value if the measurements were made in a large continuous range of frequency. In the present state of the technique, the phase method is in many respects surpassed by the pulse methods. Among these, the single photoelectron counting method presents a set of advantages not found in other methods [27,28,29, 29a]. It is often possible with this method to follow the time course of the emission anisotropy during an appreciable fraction of the fluorescence decay and is currently being applied in the determination of correlation times in a number of biological compounds [29a, 30, 30a]. The method may also be applied to the study of energy migration between a set of identical chromophores [31,32].

It is well known that energy transfer between identical molecules decrease the static anisotropy [33,34,35]. Consequently, the decay of anisotropy obtained after a flash excitation may be influenced by energy migration. One must be aware of this possibility when interpreting such measurements, and it is necessary to separate Brownian and transfer contributions [31]. A quantitative analysis of the transfer contribution may bring about detailed information on the spatial distribution of the chromophores. This principle has been applied to the study of the ethidium-DNA complex [32,36,36a].

I shall first review the principle of the experimental determination of anisotropy decay. Then the theory of Brownian depolarization will be discussed and its application to the study of macromolecules. Finally, some aspects of energy migration will be given.

II. EXPERIMENTAL DEFINITION OF THE EMISSION ANISOTROPY

A. Definitions

The state of the fluorescence polarization may be characterized by the three polarized components Ix, Iy, Iz. Their sum

$$S = Ix + Iy + Iz \tag{1}$$

is proportional to the fluorescence flux radiated in all directions. The excitation is due to the local electric field propagated by the exciting light. Then, if the exciting light is polarized linearly along OZ, one has by symmetry

$$Ix = Iy \tag{2}$$

If the exciting beam is made of natural light vibrating in the XOZ plane, then

$$Iz = Ix \tag{3}$$

By observing the fluorescence along OX, one sees

$$I_{\parallel} = Iz \qquad I_{\perp} = Iy \tag{4}$$

The anisotropy of emission [7] may be defined as

$$r = D/S \tag{5}$$

with

$$D = I_{\parallel} - I_{\perp} \tag{6}$$

According to Eqs. (1-5), the emission anisotropy for a vertically polarized exciting light is

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \tag{7}$$

and for a natural exciting light

$$r_n = \frac{I_{\parallel} - I_{\perp}}{2I_{\parallel} + I_{\perp}} \tag{8}$$

One may easily show that between the emission anisotropy r and r_n one has the relation

$$r = 2r_n$$

The use of emission anisotropy instead of the degree of polarization leads to simpler theoretical interpretations of the experiments. These parameters are related to each other by simple expressions [7].

The emission anisotropy of a solution containing several molecular species is given by the following expression [7,12]:

$$r = \Sigma r_k f_k \tag{9}$$

where r_k is the emission anisotropy of the species k, and $f_k = S_k/S$ is the fractional fluorescence intensity of the species k. All these definitions and expressions are valid for continuous excitation as well as for an excitation by an infinitely short flash.

B. Anisotropy Decay

If the fluorescence is excited by an infinitely short flash, the intensities $I_{\parallel}$, $I_{\perp}$, S, and D become time dependent. S is proportional to the fluorescence decay and, in the simplest cases, decays as a single exponential:

$$S = S_0 e^{-t/\tau} \tag{10}$$

where τ is the lifetime of the excited state.

The anisotropy decay will be defined as

$$r(t) = D(t)/S(t) \tag{11}$$

These functions are not directly measurable in a pulse fluorometer because the exciting flash is not infinitely short. One directly measures the experimental functions $i_{\parallel}(t)$ and $i_{\perp}(t)$. Let us assume that the exciting light is vertically polarized. Then one calculates [37,38]

$$\begin{aligned} d(t) &= i_{\parallel}(t) - i_{\perp}(t) \\ s(t) &= i_{\parallel}(t) + 2i_{\perp}(t) \end{aligned} \tag{12}$$

The functions D(t) and S(t) are related to these experimental functions by the following convolution integrals:

$$\begin{aligned} d(t) &= \int_0^t D(t - T)g(T)\, dT \\ s(t) &= \int_0^t S(t - T)g(T)\, dT \end{aligned} \tag{13}$$

where g(T) is the response function of the pulse fluorometer. This function essentially depends on the excitation function (time distribution of the intensity in the flash) and on the response function of the photomultiplier. The determination of this function is discussed elsewhere [27,28,29,38a]. The resolution of Eq. (13) is