Chapter 4

FLUORESCENCE: SOLUTION STUDIES

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TABLE OF CONTENTS

I. INTRODUCTION

Following absorption of a photon by a chromophore, there are a variety of pathways the electron can return to the ground state energy level from the excited state. One of these is by emission of a photon in the form of fluorescence, usually with a longer wavelength (lower energy) than the absorbed photon. It is this process, and processes that compete with this process that we shall be concerned with in this chapter. Since there are several excellent treatments of fluorescence theory and instrumentation,[1-3] only a brief description of theory will be given here, and this description will be presented so as to show the basis for the various biochemical applications that are discussed in this chapter.

A. The Franck-Condon Principle

The Franck-Condon principle states that the transition from the ground state to the excited state is essentially instantaneous compared to the time necessary for nuclear coordinates to change. This is shown schematically in Figure 1. The excitation process is an extremely rapid process, occurring in about 10^{-15} sec. Since the potential energy diagram for the singlet excited state is not completely symmetrical with the ground state, excitation from the ground state does not necessarily result in formation of the lowest energy state in the first singlet excited state. Transitions to the lowest energy level in the first singlet excited state however, occur quite rapidly (compared to emission). Emission from the lowest energy level in the singlet excited state occurs in about 10^{-9} sec. Thus the lowest energy level in the first singlet excited state represents a semistable state compared to the initial absorption state. This state may be referred to as the equilibrium excited state, as opposed to the initial state, which is frequently referred to as the Franck-Condon excited state. Since in the ground state most electrons will populate the lowest energy levels available at normal temperatures, absorption usually occurs from this state. The vibrational energy level attained in the excited state upon excitation will depend on the wavelength of the exciting light. However, the return to the ground state by fluorescence will always occur from the equilibrium excited state. A corollary of the Franck-Condon principle is that the most likely transition from the equilibrium excited state to the ground state is one where there is again no change in nuclear positions. In Figure 1, this corresponds to the transition from the midpoint of the lowest vibrational energy level of the equilibrium excited state to B. The most probable transition, in both excitation and emission results in the most intense band, either excitation or emission. In the case of emission, less likely transitions will occur to vibrational levels adjacent to AB, giving rise to weaker emission at wavelengths around the emission maximum. Hence, the shape of the emission spectrum will depend on the relative positions of the energy profiles shown in Figure 1. As a direct result of this, it can easily be seen that for a single fluorescing species, the shape of the emission spectrum is independent of the excitation wavelength. The most intense excitation band will be the Franck-Condon transition, E→F in Figure 1, followed by rapid decay into the equilibrium excited state, X-Y in Figure 1. Excitation at other wavelengths around the excitation maximum will result in transitions to other vibrational energy levels in the excited state, followed by rapid decay, again to the equilibrium excited state, X-Y. As a result, all fluorescence from the excited state occurs with a maximum governed by the transition to B, with an emission spectrum whose shape is governed by the shapes of the energy profiles of the ground state and excited state. This concept is particularly useful in establishing purity of the fluorescing species. If more than one fluorescing species is responsible for an observed emission spectrum, the shape, and emission maximum of the fluorescence will depend on the excitation

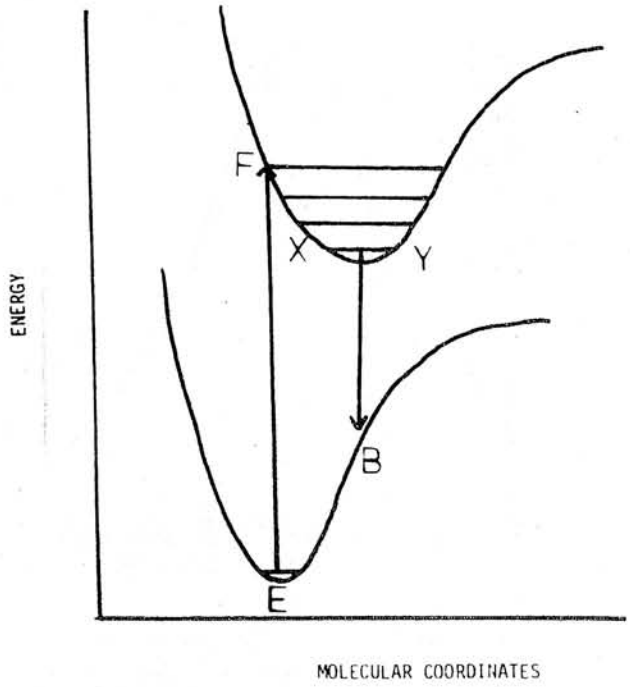


FIGURE 1. Diagrammatic representation of the Franck-Condon principle.

wavelength. This is shown experimentally in Figure 2, which shows the effects of varying the excitation wavelength on the emission spectrum of glyceraldehyde-3-phosphate dehydrogenase. Since there are a number of tryptophan residues in glyceraldehyde-3-phosphate dehydrogenase in different environments (which affects the fluorescence properties of the tryptophans, hence they can be regarded as being different fluorescing species: see later), the emission maximum shifts as the excitation wavelength is changed, indicating that the tryptophans are indeed in different environments.

B. Processes Competing with Emission

Once an electron has reached the equilibrium excited state, fluorescence is not the only route open to it. These different options are outlined in Figure 3. The energy may be dissipated, by transition into internal vibrational and rotational energy levels, as heat to solvent molecules, with no emission. Alternately, the energy may be transferred directly to a colliding, or complexed molecule, again with no emission. This process, quenching, has a number of applications in biochemical systems that will be discussed in Section V. As was discussed in the chapter on UV absorption, the most likely transition to occur upon absorption is one to a singlet excited state. There is, however, a possibility that the electron in the equilibrium excited state (singlet) can change its spin, with the resultant formation of a triplet state. Energy can then be dissipated from this triplet state by similar mechanisms to those discussed here. Emission from triplet states occurs as phosphorescence. This process is called intersystem crossing. The final process that can compete with fluorescence is resonance energy transfer, where the energy can be transferred without direct interaction to a second, acceptor molecule. Resonance energy transfer has given much information in biochemical systems and will be discussed in Section VI.

The quantum yield of the fluorescence (q) is defined as

$$q = \frac{\text{number of photons emitted}}{\text{number of photons absorbed}} \qquad (1)$$

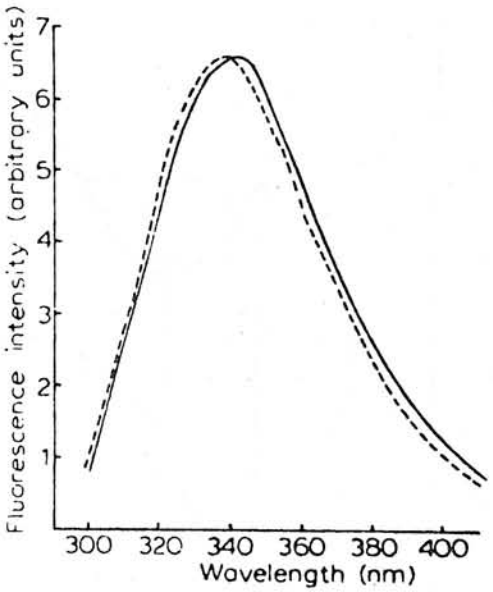


FIGURE 2. Effects of different excitation wavelengths on the emission properties of glyceraldehyde-3-phosphate dehydrogenease. The spectra are corrected for the changes in excitation intensity as the wavelength is changed. (From Bell, J. E. and Dalziel, K., *Biochim. Biophys. Acta*, 410, 243, 1975. With permission).

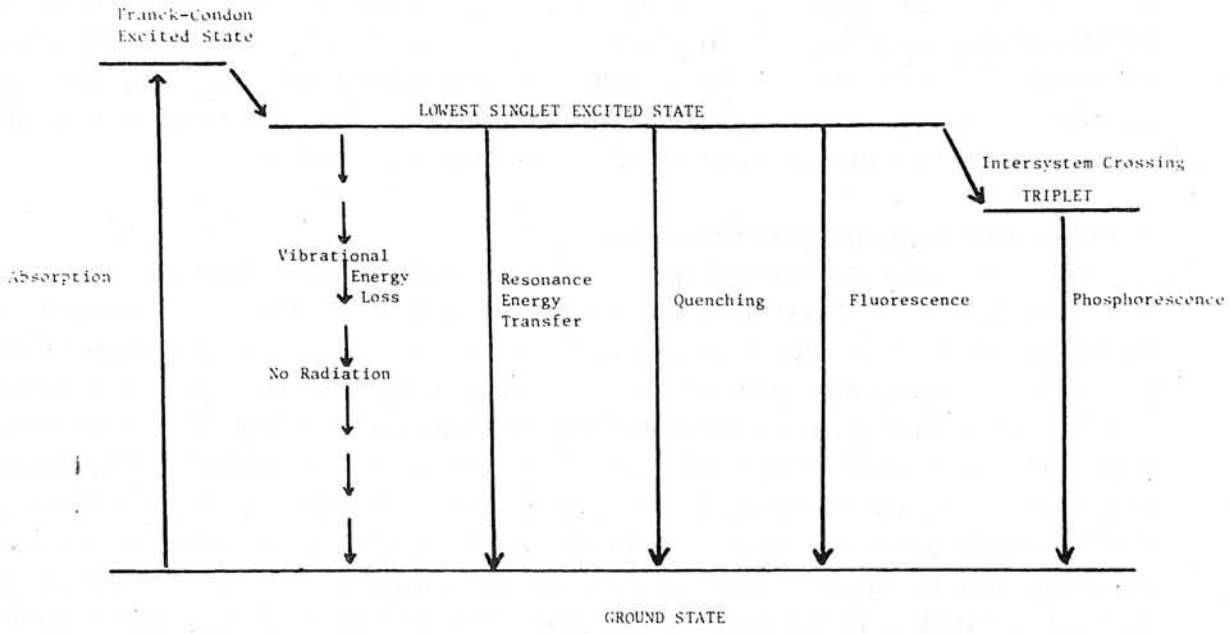


FIGURE 3. Processes that can compete with fluorescence.

thus, the quantum yield is related to the rates of the various processes we have been considering above. Experimentally q is usually determined by comparison with a standard whose quantum yield is known from direct determination.

C. Solvent Effects on Fluorescence

Since the effects of solvent on absorption processes have already been dealt with in Chapter 2, we shall confine our discussion at this point to solvent effects on fluores-

cence emission. The effects of solvent on excitation spectra are similar to those described for the effects on absorption. The most frequent observation of solvent effects is that as a fluorophore is transferred to a less polar environment, a blue shift in the emission spectrum is observed. This effect can be readily understood by reference to Figure 4. In a polar solvent (Figure 4A), solvent molecules are arranged around the ground state. On excitation however, the fluorophore dipole is changed, and in the Franck-Condon excited state, the solvent molecules are no longer arranged in the most stable configuration. However, emission occurs from the equilibrium excited state, and the solvent molecules have a chance to reorient themselves around the new excited state dipole prior to emission. Thus the excited state is stabilized by solvent interactions. In a non-polar solvent (Figure 4B) no reorientation of solvent molecules around the excited state will occur, and stabilization of the excited state does not occur. Thus, when a fluorophore is transferred from a polar to a non-polar solvent, the energy difference between the excited state and the ground state is increased due to the inability of the non-polar solvent to stabilize the excited state. This results in a *Blue* shift of the emission maximum. Similar arguments can be applied to the case where the ground state of the fluorophore has no dipole, but the excited state has an induced dipole.

D. Types of Fluorescing Molecules in Biochemical Systems

The different types of molecules whose fluorescent properties are commonly made use of in biochemical systems can be placed into three broad categories:

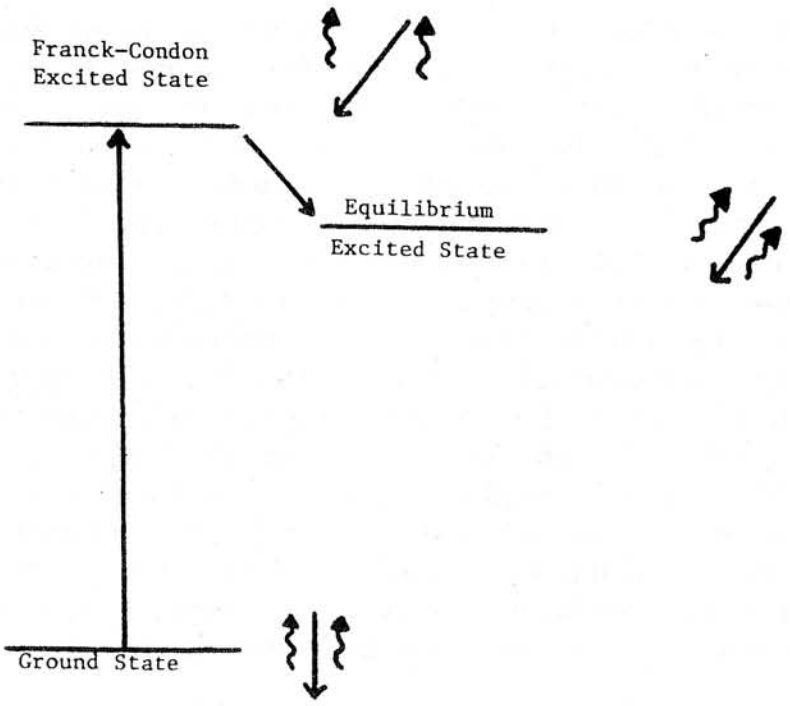
1. Natural, intrinsic fluorophores
2. Coenzymes or substrate analogs
3. Chemical modification reagents into which fluorescent moieties have been incorporated

1. Natural, Intrinsic Fluorophores

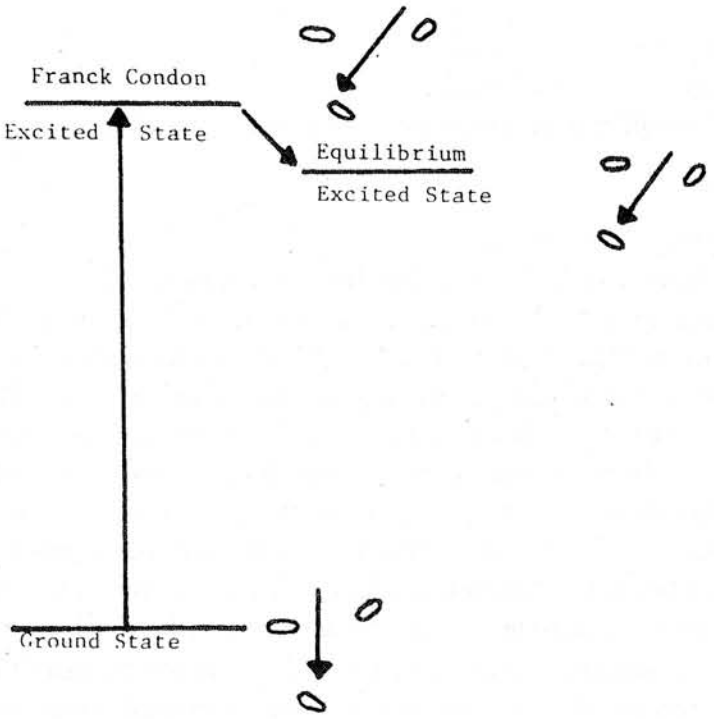
Into this category can be placed the aromatic amino acids, each of which have distinct fluorescence properties. Figure 5 shows the excitation spectra of phenylalanine, tyrosine, and tryptophan.[4] In each case, the fluorescence excitation spectrum closely follows the absorption spectrum, as should indeed be the case. The fluorescence emission spectra are shown in Figure 6. Each of the three amino acids have quite different emission spectra, phenylalanine showing maximal emission at 282 nm, tyrosine at 303 nm, and tryptophan at 348 nm. While most proteins contain all three types of residues, protein fluorescence spectra are usually dominated by tryptophan absorbance and emission, in part because tryptophan has the highest extinction coefficient of the three, and in part because resonance energy transfer from phenylalanine and tyrosine to tryptophan frequently occurs (see Section V). Nucleic acids do not in general show fluorescence at usual temperatures, and will not be discussed here. However, the Y base, which occurs in tRNA and is a guanine derivative,[5] has an excitation maximum at 315 nm and an emission maximum at about 445 nm (Figure 7). The emission maximum of $tRNA^{Phe}$, which contains the Y base next to the 3′ end of the anticodon, shows a blue shift of some 20 nm.

2. Coenzymes and Substrate Analogs

Many proteins and enzymes have coenzymes that are naturally fluorescent, for example, the dehydrogenases bind the reduced nicotinamide adenine dinucleotides (NADH and NADPH), which both fluoresce, with excitation at 340 nm and emission in the 450 nm region. Other natural coenzymes and cofactors that fluoresce are listed in Table 1 along with their excitation and emission maxima. In addition to making use of the natural fluorescence of a coenzyme or cofactor, many studies with synthetic



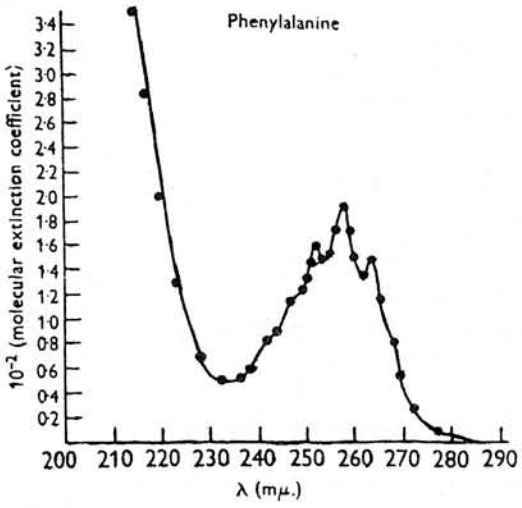
A



B

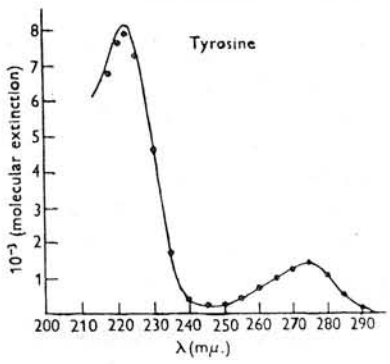
FIGURE 4. Solvent effects on fluorescence emission. (A) Polar solvent, (B) Nonpolar solvent (→) fluorophore dipole, ( ) solvent dipole (o) solvent molecules with no dipole.

fluorescent analogs of substrates or cofactors have been performed. These are too many in number to list, but several examples of this sort will be discussed in Section II. Studies of this type however, suffer from the drawback that the properties of the analog must be shown to reflect those of the true substrate or coenzyme.



A

FIGURE 5. Fluorescence excitation spectra for (A) phenylalanine, (B) tyrosine, and (C) tryptophan. The continuous lines show the optical density spectrum. (From Teale, F. W. J. and Weber, G., *Biochem. J.*, 65, 476, 1957 With permission.)



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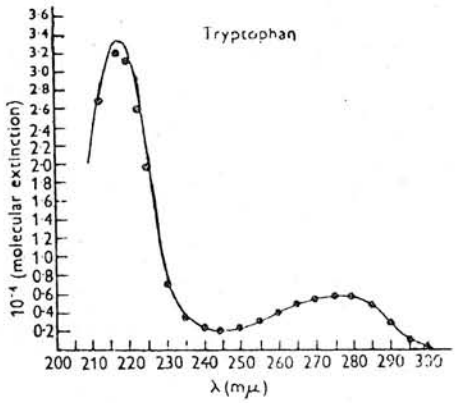

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3. Fluorescent Chemical Modification Reagents

The ability to introduce a fluorescent "reporter" group into a protein by means of chemical modification has been, and will continue to be one of the more important ways of obtaining a variety of information from the system into which it has been incorporated. While such studies are frequently used to give qualitative information about molecules they can, with proper controls, give quite specific information using the techniques discussed in later sections of this chapter. There are many types of chemical modification reagents that incorporate fluorescent groups, which show varying degrees of specificity for the modified group. By far the most popular (and most useful) are those with specificity for sulfhydryl groups. These reagents can be categorized according to the basic reagent used to modify the sulfhydryl residue. There are three major categories, maleimide based reagents, mercurial based reagents, and iodacetamide based reagents. A variety of these reagents, and others with specificity for sulfhydryl groups are given in Table 2 together with excitation and emission maxima. As can be seen, quite a wide selection of probes with a variety of absorption and emission maxima are available. The availability of such probes is of particular importance in designing resonance energy transfer experiments, which will be discussed in Section VI.

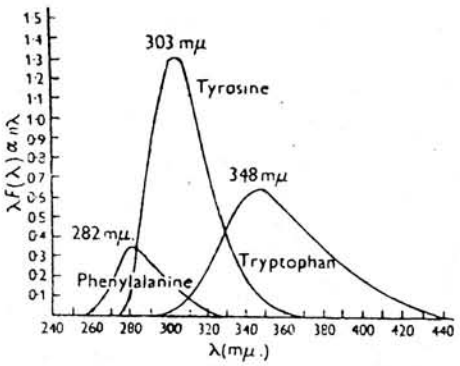


FIGURE 6. Fluorescence emission spectra of the aromatic amino acids in water. (From Teale, F. W. J. and Weber, G., *Biochem. J.*, 65, 476, 1957. With permission.)

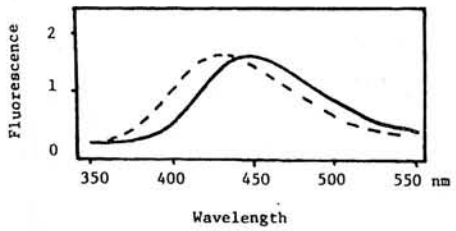


FIGURE 7. Fluorescence emission of the Y base; in solution (-)and in $tRNA^{Phe}$ (----).

Table 1
FLUORESCENT COENZYMES AND COFACTORS

	Excitation Maximum	Emission Maximum
NADH, NADPH	340	450
FAD, FMN	375	525
Chlorophyll a	420	670

Table 2
FLUORESCENT MODIFICATION REAGENTS

Fluorophore	Excitation Max. nm	Emission Max. nm
N-[p-(2-Benzoxazolyl) phenyl]-maleimide	310	370—375
4-Dimethylamino-4′-maleimido stilbene	344	480
N-(anthranilate) maleimide	320	420
N-(anthranilamide) maleimide	340—350	420—440
N-(3-Pyrene) maleimide	310—345	376—420
DNS-CYS-SHg	330—340	530
Fluorescein mercuric acetate	470	520
N-Iodoacetyl-*N*′-(5-sulfo-1-naphthyl) ethylenediamine	337	450—520
N-Iodoacetyl-*N*′-(8-sulfo-1-naphthyl) ethylenediamine	345	460—530
7-chloro-4-nitrobenzo-2-oxa-1,3-diazole	420	525
Di-dansyl-L-cystine	330	450—550

II. USES OF FLUORESCENCE SPECTRAL PROPERTIES

In this section, we will discuss how fluorescence properties such as excitation and

emission spectral properties and intensities have been used to give information on such topics as ligand binding, ligand or probe environment, and conformational changes detected using fluorescence intensity measurements or spectral properties. Fluorescence polarization, lifetimes, quenching, and resonance energy transfer will be dealt with separately.

A. Ligand Binding Studies

On binding to glutamate dehydrogenase, either NADH or NADPH show enhanced binding and shifts in emission maxima. The shifts in emission maxima will be dealt with later. Here we will describe how these changes in fluorescence intensity can be used to follow coenzyme binding to the enzyme. The rationale described here can be applied to any system where a ligand undergoes an enhancement of fluorescence on binding to protein.

Consider the case of the addition of aliquots of NADH to bovine glutamate dehydrogenase (Figure 8). Defining the following terms, we can derive an equation for the amount of bound NADH at any given point in the fluorescence titration. Let F_M be the experimentally determined fluorescence in the presence of protein at a total ligand concentration T. F_T is the measured fluorescence in the absence of protein at a total ligand concentration T. B is the concentration of bound ligand at any point in the titration. F_B is the specific molar fluorescence intensity of bound fluorophore, and F_F is the specific molar fluorescence intensity of free fluorophore.

Then, at any given point in the titration:

$$F_M = F_B.B + F_F\,(T - B) \tag{2}$$

$$= F_B.B + F_F.T - F_F.B \tag{3}$$

Since $F_T = F_F \cdot T$
We get

$$F_M = F_B.B + F_T - F_F.B$$

$$\therefore \frac{F_M}{F_T} = \frac{F_B.B}{F_T} + 1 - \frac{F_F.B}{F_T} \tag{4}$$

$$\therefore \frac{F_M}{F_T} - 1 = \frac{(F_B - F_F).B}{F_T}$$

$$\therefore B = \frac{\dfrac{F_M}{F_T} - 1}{\dfrac{F_B}{F_T} - \dfrac{F_F}{F_T}} \tag{5}$$

Substituting back $F_F \cdot T$ for F_T, we get:

$$B = \frac{\frac{F_M}{F_T} - 1}{\frac{F_B}{F_F}.T - \frac{F_F}{F_F}.T} \qquad (6)$$

Multiplying top and bottom by T we get:

$$B = \frac{T \frac{(F_M - 1)}{F_T}}{\frac{F_B}{F_F} - 1} \qquad (7)$$

Defining a fluorescence enhancement, F·E. as

$$F.E. = \frac{F_B}{F_F} \quad \text{we get}$$

$$B = \frac{T \frac{(F_M - 1)}{F_T}}{F.E. - 1} \qquad (8)$$

Hence we have an equation for the concentration of bound fluorophore in terms of T, the total ligand concentration, F_m and F_f, the fluorescences measured at a given ligand concentration in the presence and absence of protein, respectively, and a parameter F·E., which by definition is the fluorescence of the bound ligand relative to that of the free ligand. This last parameter must be determined independently. Experimentally, the fluorescence enhancement is determined by using a fixed concentration of ligand, and varying the concentration of the protein. Such an experiment is shown for glutamate dehydrogenase in Figure 9. As the protein concentration is increased progressively, more of the ligand is bound. At sufficiently high concentrations of protein, no further increase in fluorescence will be observed, indicating that all of the ligand is bound. At this point, the fluorescence enhancement can be calculated from the initial fluorescence at zero protein concentration, and the final fluorescence attained at limiting protein concentrations. Experimentally, this situation can only be achieved if one can use protein concentrations significantly higher than the dissociation constant for the ligand. Since this is not always possible, the limiting fluorescence can be determined by varying the protein concentration over a limited range and extrapolating in a double reciprocal plot to limiting protein concentrations. Once a value for the fluorescence enhancement is obtained, binding parameters for ligand binding to the protein can be determined. It should be noted that the fluorescence enhancement for a given ligand binding to protein may be affected by other ligands binding at other sites to the protein. This is shown for glutamate dehydrogenase in Table 3. As can be seen, the fluorescence enhancement in various ternary and quaternary complexes is very different from that observed in the binary enzyme-NADH complex. Unless fluorescence en-

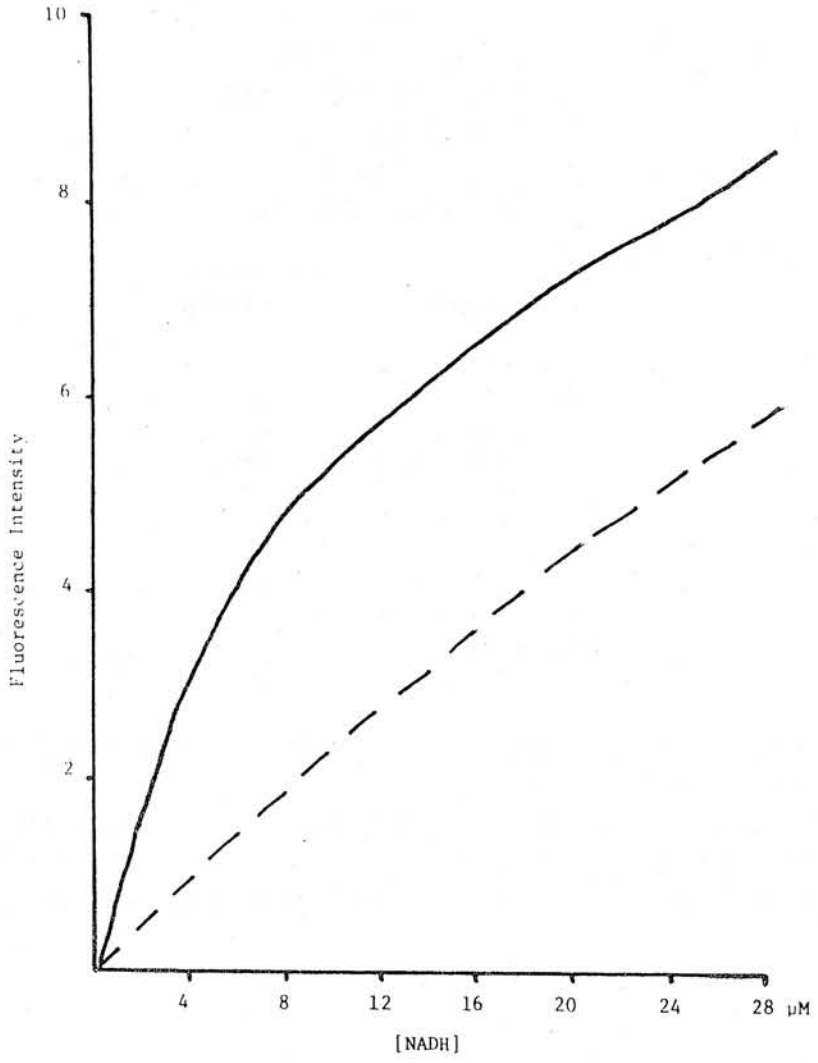


FIGURE 8. Fluorescence titration of bovine glutamate dehydrogenase with NADH; ( ) with protein; (—-) without protein.

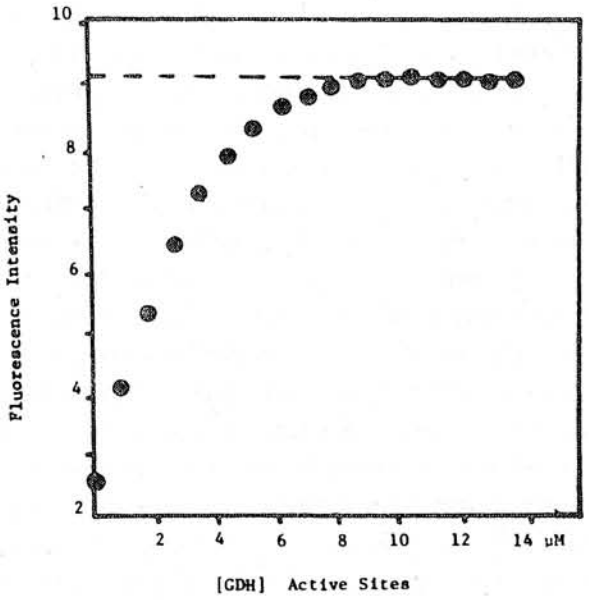


FIGURE 9. Determination of the fluorescence enhancement for NADH binding to bovine glutamate dehydrogenase.

Table 3
FLUORESCENCE ENHANCEMENTS FOR NADH BINDING TO GLUTAMATE DEHYDROGENASE

Other Ligands	Fluorescence Enhancement
A. pH 7.0	
None	2.1
Glutamate	3.47
Glutamate + ADP	3.60
Glutamate + GTP	4.2
B. pH 8.0	
None	3.6
Glutamate	3.47
Glutamate + ADP	3.13
Glutamate + GTP	4.2

hancement values are determined for the particular situation being studied, very strange artifacts may be obtained.

It might be noted that arguments similar to those presented here may be applied to the situation where a decrease in ligand fluorescence is observed upon binding to protein. A very similar equation to that derived above holds for this situation:

$$B = \frac{\dfrac{T\ (1 - F_M)}{F_T}}{1 - F.E.} \qquad (9)$$

Where of course, F.E. is no longer a fluorescence enhancement, but a quenching coefficient.

Frequently, when ligand binds to protein, a decrease in the fluorescence of the protein is observed. This quenching of protein fluorescence may also be used to determine ligand binding. There are two types of experimental situations that can occur, depending on the range of dissociation constants to be measured and protein concentrations that can be used. When a very low dissociation constant is to be determined a very different experimental approach applies, and can yield information with fewer uncertainties than if weaker binding is to be studied. In studies on rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, it was observed that at relatively high protein concentrations (Figure 10) quenching of protein fluorescence was linear with coenzyme concentration up to a ratio of coenzyme to protein of about 2. Beyond this point the quenching was no longer linear. From this linear region of quenching the extent of quenching induced by the binding of each coenzyme molecule could be determined.[6] Once this parameter is obtained, much lower protein concentrations can be used where ligand binding is not stoichiometric with ligand concentration. Such titrations (Figure 11) can then be used to obtain binding data for coenzyme binding to glyceraldehyde-3-phosphate dehydrogenase, since the amount of quenching produced by each molecule of coenzyme binding has been determined. However, in many instances, where weak binding is being observed, it is difficult to attain experimentally a situation where quenching of protein fluorescence is stoichiometric with ligand added, and hence the

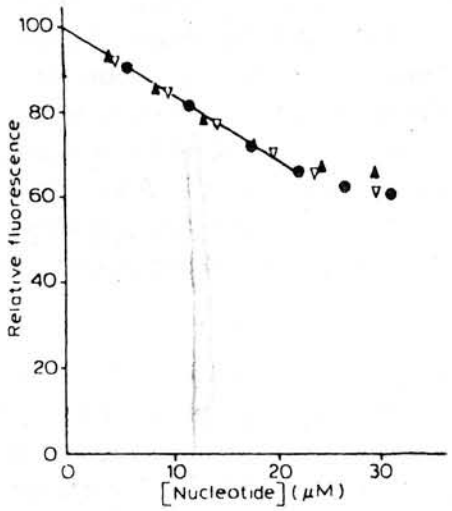


FIGURE 10. Quenching of glyceraldehyde-3-phosphate dehydrogenase fluorescence by NAD+ at high protein concentrations (From Bell, J. E. and Dalziel, K., *Biochim. Biophys. Acta*, 391, 249, 1975. With permission.)

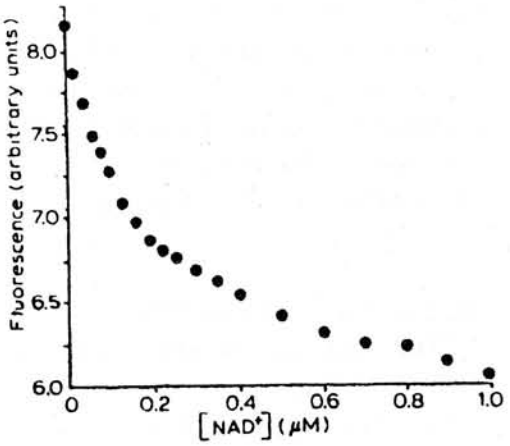


FIGURE 11. Quenching of glyceraldehyde-3-phosphate dehydrogenase fluorescence by NAD+ at low protein concentrations. (From Bell, J. E. and Dalziel, K., *Biochim. Biophys. Acta*, 391, 249, 1975. With permission.)

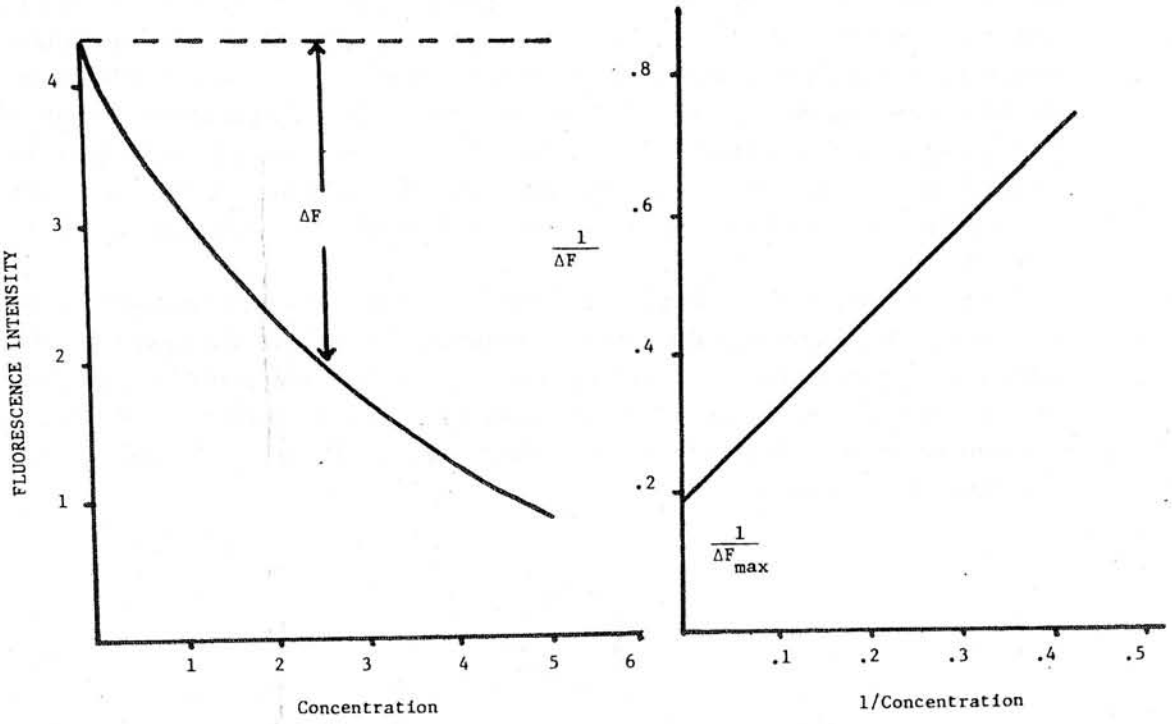


FIGURE 12. A representation of ligand produced fluorescence quenching in a "low affinity situation". A. Fluorescence vs. ligand concentration B. Double reciprocal plot of "data" from A. Showing determination of maximum quenching.

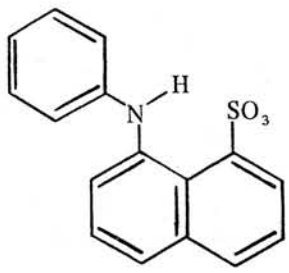
ligand-dependent parameter described above cannot be obtained. However, ligand binding can still be observed and followed in such a situation with several limiting assumptions. Figure 12 illustrates a typical experimental situation. As ligand concentration is increased at a fixed protein concentration, a gradual decrease in fluorescence is observed. From such data, it is not possible to calculate either how much quenching

is produced per ligand bound, or how much of the fluorescence is quenched at saturating concentrations of ligand. The latter parameter can be obtained from a double reciprocal plot of $1/\Delta F$ vs. $1/[L]$ if it is assumed that each molecule of ligand bound produces an equal amount of quenching. Once this parameter is obtained, the amount of ligand bound at any point in the titration can be calculated if it is assumed that at maximum quenching all of the protein sites contain ligand — hence one must assume a number of binding sites per protein molecule, and no information about this parameter can be obtained from the binding study. However, information about affinity is obtained.

B. Environmental Probes

The intensity and spectral properties of a fluorophore may be used to give information concerning the environment of the fluorophore. Intrinsic fluorescence properties of a macromolecule may be used to infer such information. The existence of tryptophan residues in different environments in glyceraldehyde-3-phosphate dehydrogenase has already been discussed. The observation (Figure 2) that the emission maximum of the tryptophan fluorescence shifts to longer wavelengths when the excitation wavelength is changed from 280 nm to 295 nm indicates that some of the tryptophan residues are in a more exposed, more polar environment than the others. Another instance we have already mentioned here is the effect of ligands on the enhanced fluorescence of NADH upon binding to glutamate dehydrogenase. The emission spectra of free NADH, NADH bound to enzyme, and NADH bound to enzyme in the presence of various substrate analogs are shown in Figure 13. Free NADH has a broad emission spectrum, centered at 450 nm. On binding to the enzyme however, an enhanced fluorescence, and a shift in the emission maximum of the emission spectrum is affected by the various substrates, or substrate analogs, with L-2-hydroxyglutarate, which has been proposed as a transition state analog, giving the greatest enhancement and the largest blue shift in emission, indicating that in the enzyme-NADH-L-2-hydroxyglutarate complex the coenzyme is in a highly hydrophobic environment compared to free NADH.

Noncovalently bound fluorescent probes of the *N*-arylaminonapthalenesulfonate-type have found considerable use in biochemical systems.[7] Because of their chemical nature such probes bind to "hydrophobic" sites on many proteins, and their fluorescent properties have been taken as indications of local polarity.[8] Perhaps the most popular of such probes is 8-anilino-1-naphthalenesulfonic acid (ANS), which has the structure shown below.



When ANS is bound to UDP-galactose 4-epimerase[9] (Figure 14) a considerable enhancement in fluorescence and blue shift in emission maximum is observed, indicating binding to a hydrophobic pocket in the enzyme. In this system, ANS was shown to bind competitively with nucleosides and nucleotides, indicating that the binding site for nucleotides was hydrophobic in nature. Studies with ANS binding to either holo-,

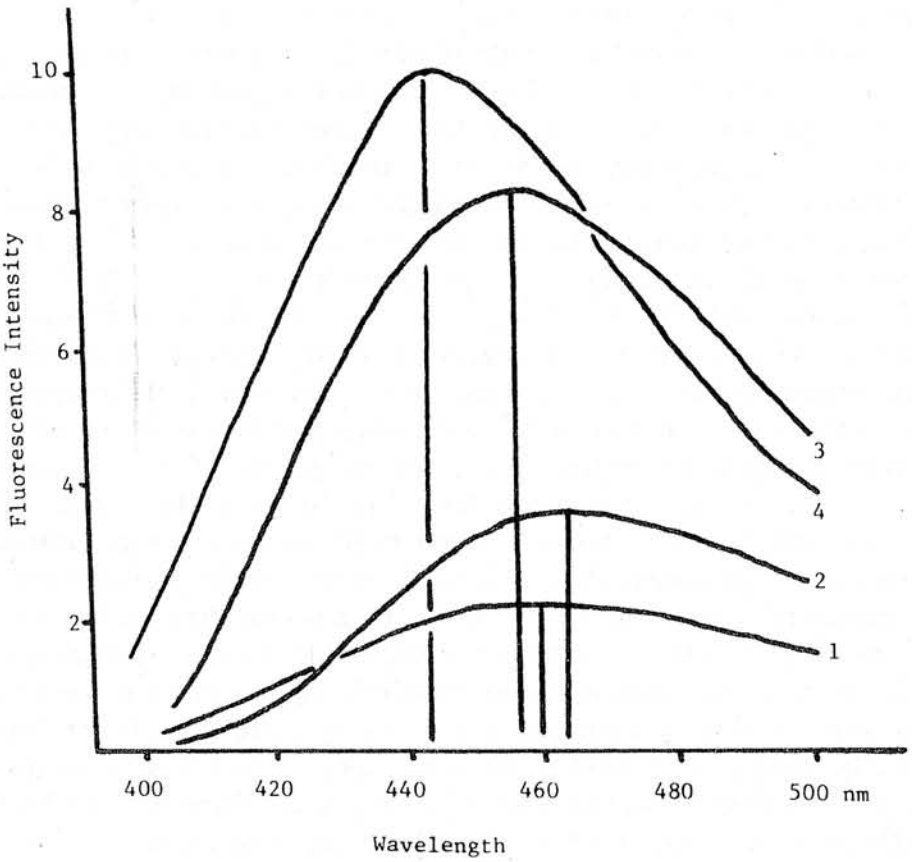


FIGURE 13. Emission spectra for GDH — coenzyme complexes. Excitation at 340 nm. 1. Free NADH, 2. Enzyme-NADH, 3. Enzyme-NADH-L-glutamate, and 4. Enzyme-NADH-L-3-hydroxyglutarate.

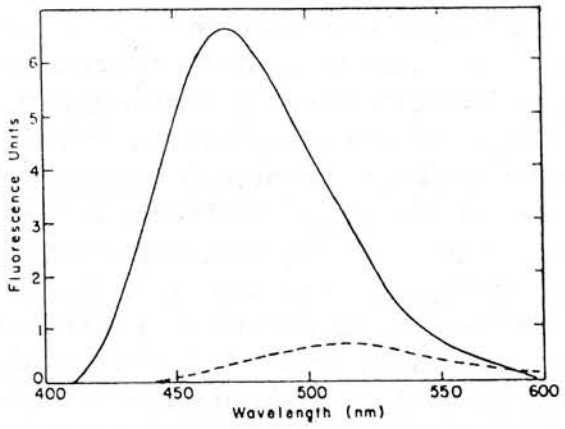


FIGURE 14. Fluorescence emission spectra of ANS (---) and epimerase-NAD-ANS complex (—). Excitation at 375 nm. (Reprinted with permission from Wong, S. S. and Frey, P.A., *Biochemistry*, 17, 3351, 1978. Copyright by the American Chemical Society.)

or apo-phosphorylase b[10] were used to show both differences in the microenvironment of the ANS between the two forms of the enzyme, and the effects of other ligands on the ANS fluorescence. The emission maximum for ANS fluorescence was found to shift from 470 nm in the apoenzyme, to 490 nm in reconstitution with pyridoxal 5′-

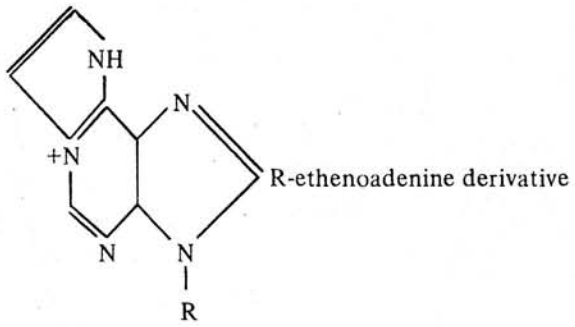
phosphate. In addition, the fluorescence quantum yield of the bound ANS calculated by measuring the emission spectrum of ANS in the presence of varied concentrations of either apo- or holo-enzyme, Figure 15, and extrapolating to "saturating" protein concentrations, was found to be three times higher in the apoenzyme.

While this type of study can be, and has been used to look at hydrophobicity of ligand binding sites as in the case with UDP galactose-epimerase above, and may give some information about differences in the probe binding site, as in the example of phosphorylase above, this type of approach is fraught with difficulties, both in experimental design and interpretation. To be sure that observed changes in intensity or spectral maxima are due to real changes in these properties of the bound probe, and not due to different extents of binding between the states being compared, it is essential to assure that the extent of binding is not being changed, or is at least being accounted for. With noncovalent probes, this is not always easy. Many of these problems are overcome by the use of covalently bound probe molecules, which will be discussed later. Interpretation of changes induced in probe fluorescence, whether covalent or noncovalent is not always clear. Where the probe can be shown to bind competitively with respect to a particular ligand, it can be assumed that the spectral characteristics of the probe reflect the environment of the ligand; however this need not be the case. The ligand could be inducing a conformational change in the protein that prevents the probe from binding to a separate site. Thus, interpretation is not clear. If the probe and the ligand can both bind at the same time, one is left with the problem of where is the probe molecule binding, and what exactly do changes in the probe fluorescence indicate other than that a conformational change is occurring.

Many of these problems were overcome in studies with oligomeric dehydrogenases,[11,12] and the principle behind these studies is widely applicable. The main problem in interpretation of conformational changes detected using fluorescence probes is knowing where the change being detected is occurring. In models of cooperativity, the major problem in discriminating between models, especially for negative cooperativity, is detecting conformational changes induced across subunit interfaces. While this has been achieved for hemoglobin[13,14] using crystallography, this is difficult to achieve in solution. However, using the fluorescence properties of the natural coenzymes NADH and NADPH for glutamate dehydrogenase, it was shown that NAD^+ or $NADP^+$ induced conformational changes in the hexamer of the enzyme, which occurred across subunit interfaces. The approach depends on two points. First, the fluorescence properties of the bound NAD(P)H reflects the conformation of the NAD(P)H binding site, and, secondly that oxidized and reduced coenzyme cannot be bound to the same binding site at the same time. The fluorescence properties of the bound reduced coenzyme must be established by titrating a fixed concentration of the reduced coenzyme (fluorescence probe) with enzyme to obtain a fluorescence enhancement for the bound coenzyme. Such titrations are then repeated in the presence of the ligand (in this case oxidized coenzyme), which is thought to induce conformational changes. The fluorescence enhancement obtained in the presence of the second ligand is thus a monitor of the environment of the reduced coenzyme (fluorescent probe). In the case of glutamate dehydrogenase, it was found that when the hexamer was on average half saturated with oxidized coenzyme, the fluorescence properties of the bound reduced coenzyme (fluorescent probe) abruptly changed, (Figure 16) indicating that the environment of the probe had been changed. Since in this particular case the probe (the reduced coenzyme) and the ligand (oxidized coenzyme) must bind at the same site in each subunit, it is apparent that the changes in fluorescence observed in the bound probe (reduced coenzyme) must be caused by ligand binding to subunits other than that to which the probe is bound.

Using the natural fluorescence of a substrate or effector has the obvious advantage

of not introducing a potential perturbant into the system. However, this is not always possible. It may be possible to use flourescent analogs of the substrate or ligand as probe molecules. With such analogs it is essential to establish that the properties that are being studied are not perturbed by modification of the ligand to incorporate the fluorescent group. A particularly useful series of coenzyme analogs are those based on 1:N^6-ethanoadenine[15,16] whose structure is shown below:



These derivatives have excitation maxima at about 305 nm and emission maxima at about 415 nm, and have been shown to be catalytically active in a number of systems. These include the use of 1:N^6-ethanoadenosine triphosphate with myosin ATPase,[17] the incorporation of 1:N^6-ethanoadenosine into poly(A)[18] and yeast tRNA^{Phe},[19] and the inclusion into FAD.[20] The NAD$^+$ analog, ethano-NAD$^+$ has been used in the glyceraldehyde-3-phosphate dehydrogenase system to examine induced conformational changes.[21]

We have discussed here how use may be made of the fluorescence properties of either natural ligands or fluorescent analogs of ligands to obtain information about binding site hydrophobicity or conformational changes occurring in binding sites. In addition to these approaches information can also be obtained from studies of the intrinsic protein fluorescence. As mentioned earlier, ligand binding often produces a quenching of the intrinsic protein fluorescence, and binding data can be obtained from such studies. There is however, the potential of extracting more information from such observations. We have discussed how the fluorescence emission spectrum of a protein will contain contributions from tryptophan residues in a variety of environments. The quenching effects produced by a ligand can be categorized by which class or classes of tryptophans are affected by ligand binding. Experimentally, this is done by measuring the quenching observed at a particular emission wavelength with various exciting wavelengths. If the residue whose fluorescence is most affected by ligand interaction is in a more hydrophobic environment than the remaining residues, a comparatively greater degree of quenching will be observed at wavelengths to the "*blue*" side of the usual excitation maximum than would be observed to the "*red*" side of this wavelength. In effect, a "quenching spectrum" can be constructed showing what the environment of the "quenched" residue is. This approach has been successfully applied to glyceraldehyde-3-phosphate dehydrogenase,[12] where the "quenching spectrum" is seen to change (Figure 17) depending on the saturation of the tetramer with coenzyme. At low degrees of saturation, the maximal quenching occurs at about 265 nm indicating that at this point either blue shifted tryptophan residues, or possibly tyrosine or phenylalanine residues are being affected by binding of coenzyme. At higher degrees of saturation, the quenching maximum is shifted to higher wavelengths, indicating that the enzyme has undergone a conformational change such that different residues (or the same residues, but in different environments) are being affected by coenzyme binding. Similarly, the intrinsic tryptophan fluorescence of free ribosomal proteins has been used

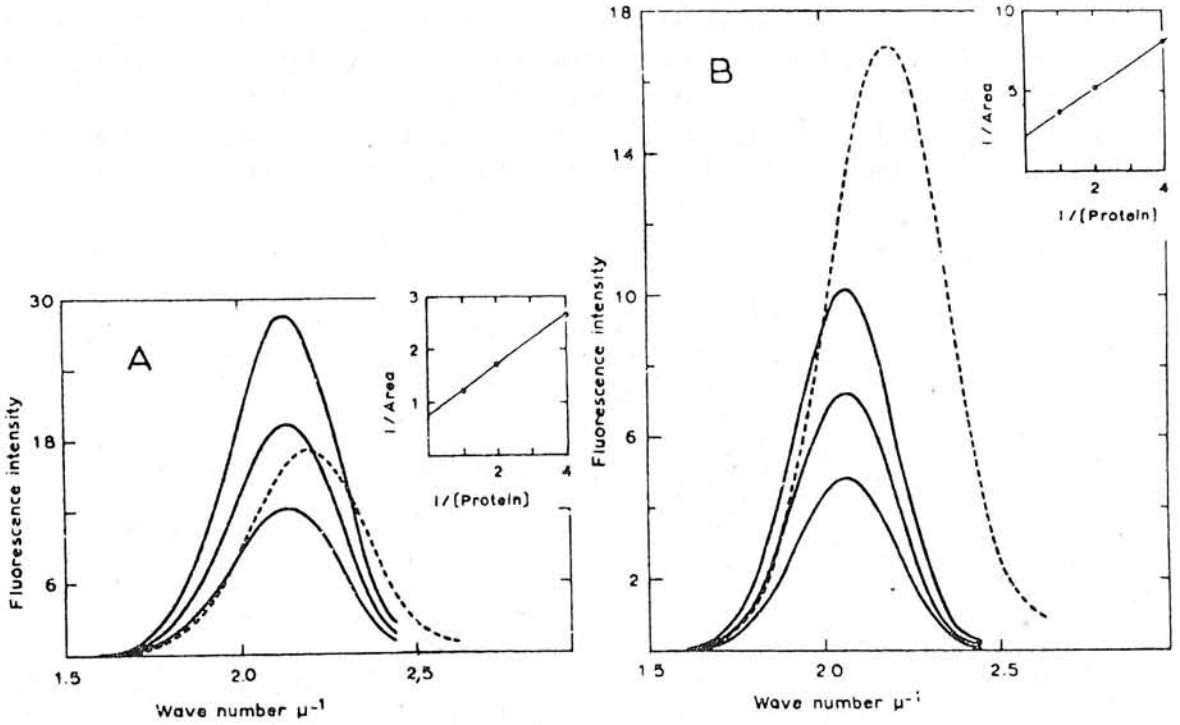


FIGURE 15. Emission spectra for ANS complexes of A) apophosphorylase, and B) phosphorylase at protein concentrations of 4, 2, and 1 mg/ml. Areas under the curves were measured and, by means of reciprocal plots (inserts) extrapolated to infinite protein concentration. (Reprinted with permission from Klunsoyr, L., *Biochemistry*, 13, 1751, 1974. Copyright by the American Chemical Society.)

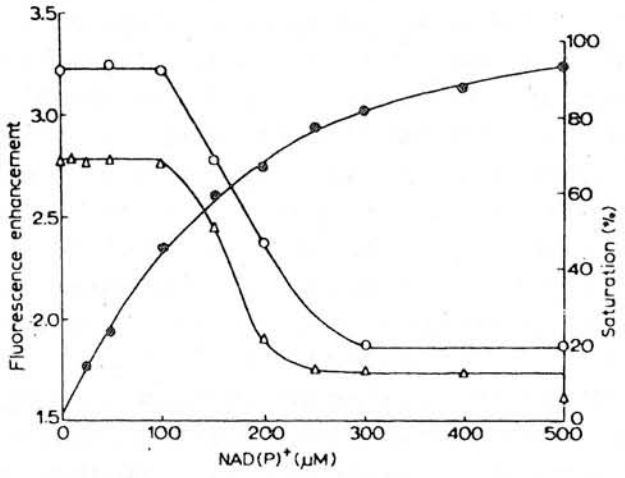


FIGURE 16. Effects of NAD⁺ (Δ-Δ), or NADP⁺ (o-o) on the fluorescence enhancement of bound NADH in the presence of 0.2 *M* glutarate. The saturation curve for NAD⁺ binding is also shown (●-●). (From Bell, J. E. and Dalziel, K., *Biochim. Biophys. Acta*, 309, 237, 1973. With permission.)

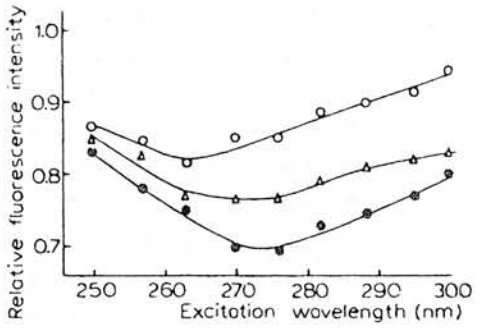


FIGURE 17. Quenching of protein fluoresence at different excitation wavelengths by NAD⁺. The relative fluorescence intensity is the ratio of the measured intensity in the presence of 2.9 μM (NAD⁺ (o-o), 5.8 μM (Δ-Δ) and 11.5 μM NAD (●-●), and that of the apoenzyme (2.9 μM). In all cases emission was measured at 342.5 nm. (From Bell, J. E. and Dalziel, K., *Biochim. Biophys. Acta*, 410, 243, 1975. With permission.)

to follow organization of the proteins into 30S subunits.[22] The emission spectrum of the free proteins is blue shifted relative to that of free tryptophan; however, a further blue shift is observed when the proteins are incorporated into ribosomes, and the fluorescence was sensitive to procedures that are known to affect the ribosome structure such as magnesium depletion.

Covalently localized fluorescent probes have been used in a wide variety of systems

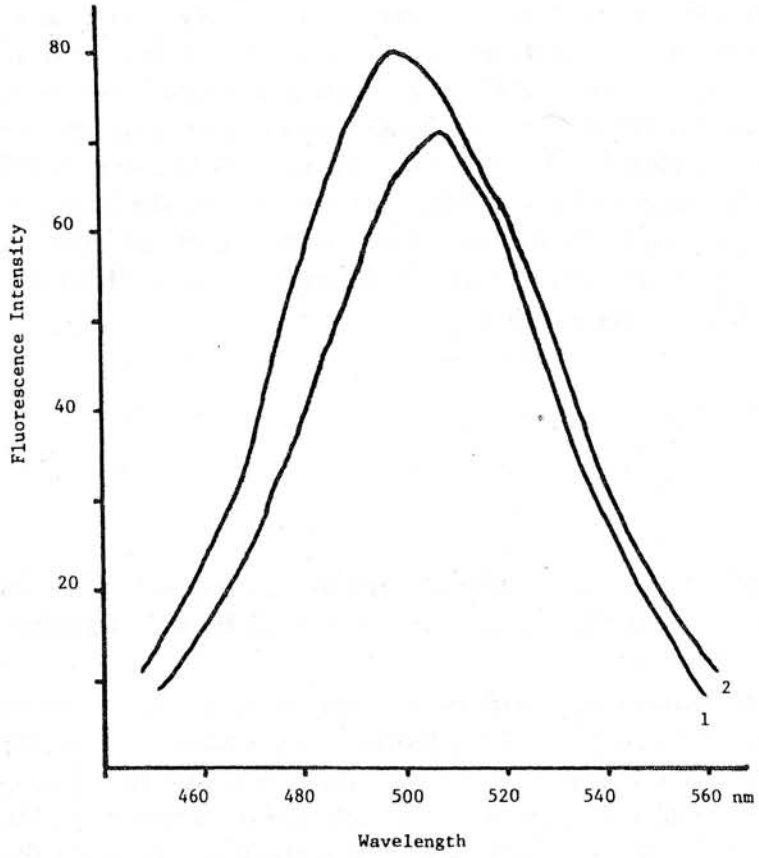


FIGURE 18. Emission spectra of NBD-labeled glyceraldehyde-3-phosphate dehydrogenase in the absence (1) or presence (2) of saturating concentrations of NAD^+. Excitation was at 420 nm.

to give information on probe environment and conformational changes induced in the probe environment by ligand binding. The key to the interpretation of such studies is "probe environment". The fluorescent properties of the probe reflect the probe environment, and measured changes are the result of changes in the probe environment. If the localization and uniqueness of labeling with the probe can be established, useful information can be obtained. If, on the other hand, the system contains more than one class of label, and nothing is known about the location of the label, little information can be obtained other than indications that conformational changes are taking place.

Using the reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) to modify sulfhydryl residues in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase[23] an active, fluorescently labeled enzyme was obtained. When coenzyme bound to the modified enzyme a slight increase in fluorescence intensity and a 4 to 5 nm blue shift in the emission maximum was observed (Figure 18). When these fluorescence changes were followed as a function of coenzyme binding, it was found that the fluorescence probe monitored changes induced by coenzyme binding.

While such studies have been used to infer conformational changes induced by ligands, the most important uses of covalently incorporated fluorescent probes has come in polarization studies or resonance energy transfer studies, and these applications will be discussed in the appropriate sections.

III. POLARIZATION

A. Introduction

A single ray of light can be regarded as being characterized by the orientation of its

electric vector at some angle θ as illustrated in Figure 19A. A beam of light is thus characterized by the angular distribution of its electric vectors with the two extremes being shown in Figures 19B and C. These two possibilities can be distinguished by viewing the beams through a polarizer, which blocks passage of light when it is aligned perpendicular to the electric vector of the light rays. If the electric vectors were randomly oriented (all values of θ possible), the intensity of the light passing through a polarizer will remain constant whatever the orientation of the polarizer. If, however, θ = zero or $\theta = 90°$ the transmission will depend on the angle of the polarizer. The polarization of fluorescence is defined as:

$$P = \frac{F_{11} - F_{\underline{1}}}{F_{11} + F_{\underline{1}}} \qquad (10)$$

Where F_{11} and $F_{\underline{1}}$ refer to the vertical and horizontal intensity components of the fluorescence. When all possible angles of θ are allowed both components are equal and P = zero.

In an absorbing molecule, maximum absorption occurs when the absorption dipole is parallel to the electric vector of the light. The probability of absorption is proportional to $\cos^2\theta$ where θ is the angle between the dipole and the electric vector component of the light. As a result, when $\theta = 0°$ absorption is maximal. Similarly emission probability is proportional to $\sin^2 E$, where E is the angle between the dipole and the electric vector of the emitted light. Since the number of molecules that emit at a given angle is proportional to the probability of emission at that angle, the intensity of emission is also proportional to $\sin^2 E$.

In our discussion here, we will assume that molecules are being excited by polarized light. Excitation takes place in effect instantaneously as discussed before. If emission took place instantaneously as well, the orientation of the emitted light would depend solely on the orientation of the dipole of the excited state. However, a finite amount of time passes prior to emission. In this time, the molecule has a chance to reorient itself, and the orientation of the emitted light will depend on the degree of molecular reorientation that takes place. If the molecule has a chance to totally randomize its orientation prior to emission both components F_{11} and $F_{\underline{1}}$ will be equal as discussed above and the polarization will be zero. If no molecular reorientation can take place, the polarization will be of course be maximal.

B. The Perrin Equation

The fluorescence intensity can be resolved into three components, whose electric vectors are parallel to the mutually perpendicular axes X, Y, and Z (Figure 20A). Since the intensity is proportional to the square of the electric field strength, the maximum intensity of each component is $\cos^2\theta_i$, where θ_i is the angle between the oscillator and the electric vector of that component. If however, each emission oscillator OE rotates over a certain angle B (Figure 20B) prior to emission, the polarization will clearly be different from the case where the oscillator is fixed.

The equation for a fixed oscillator, Equation 11

$$\frac{1}{P_o} - \frac{1}{3} = \frac{2/3}{1/4\ (3\ \overline{\cos^2\alpha} - 1)\ (3\ \overline{\cos^2\lambda} - 1)} \qquad (11)$$

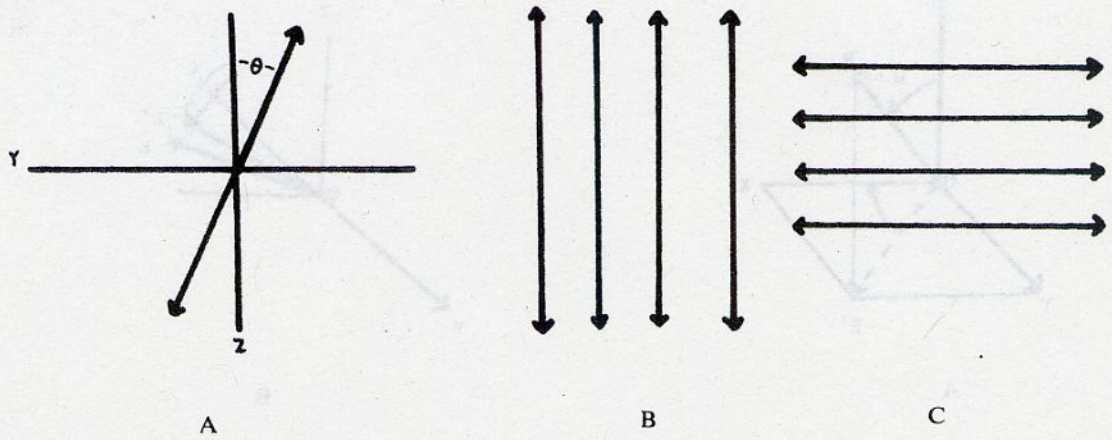


FIGURE 19. Schematic representation of polarized and unpolarized light.

has been derived previously.[2] When the emission oscillator rotates it is necessary to add the term (3/2 $\cos^2\beta$-1) to the above equation, giving Equation 12

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} \quad \frac{1}{3}\right) \times \left(\frac{2}{3\cos^2\beta - 1}\right) \tag{12}$$

Where P_o is the polarization in the absence of rotation, and P is the polarization if rotation takes place. The overall rotation, and thus $\overline{\cos_2 B}$ depends on how far a given oscillator can rotate in a given time interval, and on how many oscillators emit at a given time, t, after excitation.

Since, for a molecule undergoing Brownian motion

$$\beta^2_{\delta t} = \frac{4\overline{E}\,\delta t}{F_r} \tag{13}$$

Where $\beta^2_{\delta t}$ is the square of the average angular displacement during time δt, $\overline{E}$ is the average kinetic energy possessed by the molecule during δt, and F_r is the frictional coefficient of the rotating species. Since δt is small, $\beta^2_{\delta t}$ is small and $\sin^2\beta_{\delta t} = \beta^2_{\delta t}$, and

$$1 - \overline{\cos^2\beta}\delta t = 3\,\overline{E}\,\delta t/fr \tag{14}$$

since the rotation of a dipole occurs around two axes, $\overline{E} = KT$ with,

$$\overline{\cos^2\beta}_{\delta t} = 1 - 4KT/fr$$

which can be rearranged to give Equation 15

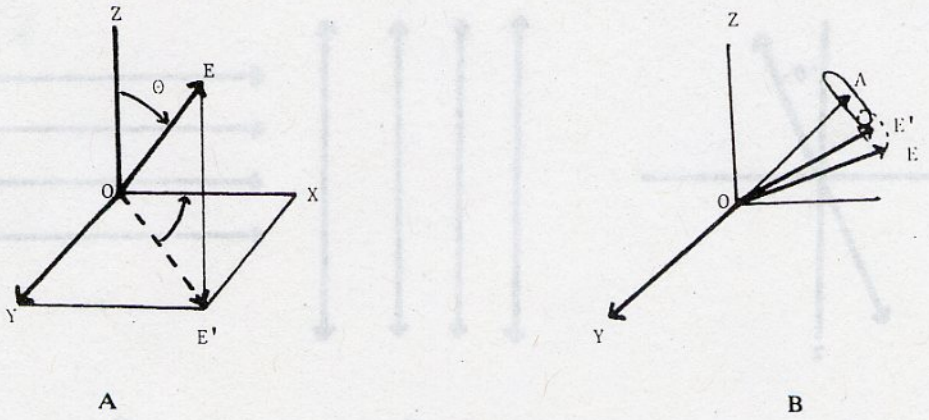


FIGURE 20. (A). Representation of the components of fluorescence. (B) Representation of the effects of oscillator rotation in fluorescence polarization.

$$\frac{3\overline{\cos^2\beta}\,\delta t - 1}{2} = 1 - 6KT\delta t/fr \qquad (15)$$

Since for each time interval δt a discrete displacement $\cos^2\beta\delta t$ occurs, the rotation over a period of time t can be described as $\cos^2\beta$, and there are $t/\delta t$ discrete rotations. This situation may be described by Soleillet's equation,[24] Equation 16.

$$\left(\frac{3\cos^2\beta_t - 1}{2}\right) = \left(\frac{3\cos^2\beta\delta t - 1}{2}\right)^{t/\delta t} \qquad (16)$$

Hence, substituting Equation 15 into Equation 16 we get:

$$\left(\frac{3\cos^2\beta_t - 1}{2}\right) = \left(\frac{1 - 6KT\delta t}{fr}\right)^{t/\delta t} \qquad (17)$$

When $\delta t/t \ll 1$, expansion of the exponential gives

$$\left(\frac{3\overline{\cos^2\beta t} - 1}{2}\right) = e^{-6KTt/fr} \qquad (18)$$

$$\therefore \quad \overline{\cos^2\beta t} = 1/3 + 2/3\, e^{-6KTt/fr} \qquad (19)$$

Defining a constant ϱ, the rotational relaxation time of the rotation

$$\rho = \frac{fr}{2KT} = \frac{8\pi\pi I^3}{2KT}$$

Where n is the viscosity, r is the radius of the equivalent sphere, T is the temperature, and K is the Boltzman constant. With this definition, Equation 19 becomes:

$$\overline{\cos^2\beta_t} = 1/3 + 2/3\, e^{-3t/\rho} \qquad (20)$$

Since fluorescence emission is an exponential decay process, molecules will be emitting at different times, and thus emission will occur after different degrees of rotation. The final average rotation will be weighted according to the contribution each rotation makes to the total fluorescence intensity. Therefore,

$$\overline{\cos^2\beta} = \int_o^\infty f(t)\cos^2\beta_t\, dt \qquad (21)$$

For an exponential decay, the intensity I_t at time t is:

$$I_t = I_o e^{-t/\Upsilon}$$

Where T is the fluorescence lifetime.

$$\Upsilon = \frac{\int_o^\infty t\ I_t dt}{\int_o^\infty I_t dt}$$

The total fluorescence is:

$$It = \int_o^\infty I_o e^{-t/\Upsilon}\, dt$$

the fraction of the total fluorescence f (t) being emitted at a given time t is:

$$f(t) = \frac{I_t}{It} = \frac{I_o e^{-t/\Upsilon}}{\int_o^\infty I_o e^{-t/\Upsilon} dt} = e^{-t/\Upsilon} \qquad (22)$$

Substituting Equation 22 and Equation 20 into Equation 21, we get:

$$\overline{\cos^2\beta} = \int_o^\infty \left(\frac{e^{-t/\Upsilon}}{\Upsilon}\right)\left(\frac{1}{3} + \frac{2}{3}\, e^{-3\ t/\rho}\right) dt$$

which, rearranged gives:

$$\overline{\cos^2\beta} = \frac{1}{3\Upsilon}\int_o^\infty \left(e^{-t/\Upsilon} + 2e^{-t(3/\rho + 1/\Upsilon)}\right) \tag{23}$$

Integration of Equation 23 gives:

$$\overline{\cos^2\beta} = \frac{1}{3} + \frac{2}{3\Upsilon}\left(\frac{1}{3/\rho + 1/\Upsilon}\right) = \frac{1}{3} + \frac{2}{3}\left(\frac{\rho}{3\Upsilon + \rho}\right) \tag{24}$$

Substituting Equation 24 into Equation 12 gives, after simplification:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(\frac{2}{3\left(\frac{1}{3} + \frac{2}{3}\left[\rho/\left(3\Upsilon + \rho\right)\right]\right) - 1}\right)$$

$$= \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(1 + \frac{3\Upsilon}{\rho}\right) \tag{25}$$

Since

$$\rho = \frac{8\pi\eta r^3}{2\,KT}$$

We get Equation 26

$$\frac{1}{P} - \frac{1}{3} = \frac{1}{P_o} - \frac{1}{3}\quad 1 + \frac{3\Upsilon KT}{4\pi\eta r^3} \tag{26}$$

Since the molecular volume V_o of a sphere is $4/3\ \pi r^3$ we get:

$$\frac{1}{P_o}\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(1 + \frac{KT\Upsilon}{V_o\eta}\right) \tag{27}$$

Thus, from Equation 25 and Equation 27 we get the usual form of the Perrin equation, Equation 28.

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(1 + \frac{3\Upsilon}{\rho}\right) = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(1 + \frac{RT\Upsilon}{\eta V_o}\right) \tag{28}$$

where: P is the observed polarization, P_o is the intrinsic polarization (at infinite viscosity) R is the universal gas constant, T is the temperature, V_o is the volume of an equivalent sphere, ϱ is the rotational relaxation time, η is the viscosity of the medium (in this case the microviscosity of the fluorophore environment), and T is the lifetime of the fluorophore. Thus we can easily see that the factors affecting the observed polarization are the microviscosity, the fluorescent lifetime, and the rotational relaxation time of the fluorescent species.

C. Experimental Uses of Fluorescence Polarization

From the above discussion, it is apparent that the polarization of a fluorophore depends on the environment of the fluorophore, and the size of the molecule the fluorophore is attached to, as well as to the emission properties of the fluorophore itself. It is from these properties that fluoroscence polarization derives its usefulness in biochemical systems.

1. Interaction of Small Molecules with Macromolecules

Consider the interaction of a low molecular weight fluorophore with a macromolecule. As discussed earlier, the fluorescence properties of the bound fluorophore may be sufficiently different from those of the free fluorophore that binding can be followed experimentally by monitoring these fluorescence changes. However, in certain cases there may be no significant change in fluorescence intensity at a particular wavelength, making this approach worthless. The small molecule will however have a characteristic polarization free in solution, which will depend primarily on the lifetime of fluorescence emission (molecules with very short lifetimes will have disproportionately high polarizations, for example NADH[25]). When the small molecule binds to a macromolecule, its rate of rotation in solution will now be characterized by the rate of rotation of the macromolecule, which being much larger than the small molecule will have a larger rotational relaxation time, resulting in an increase in the observed polarization. Thus, the binding of the small molecule to the macromolecule may be followed by polarization changes. This approach has been used to follow the binding of NADH to glutamate DH.[26] Polarization measurements have also been used to show that reduced coenzyme was bound to glyceraldehyde-3-phosphate dehydrogenase when the intensity and emission maximum of the bound NADH was very similar to that of free NADH.[12]

2. Interaction of Macromolecules

In many instances the interaction between proteins has been monitored using fluorescence polarization measurements. In general, one protein is fluorescently labeled covalently, and the polarization measured in the presence and absence of a second protein. If complexation between the two proteins occurs, the measured polarization will increase due to the increased rotational relaxation time of the complex relative to that of the single protein. Obviously for maximum sensitivity, the smaller of the two proteins should be fluorescently labeled. This approach was utilized in studies on lactose synthase,[27] where the polarization of dansylated αlactalbumin (mol. wt. 14,000 daltons) was studied as a function of the presence or absence of the substrates for the reaction, *N*-acetylglucosamine, glucose, UDPgalactose, Mn^{2+}, or various combinations. In the absence of substrates, the polarization in the presence of the transferase was the same as with DNA-αlactalbumin alone. When *N*-acetylglucosamine, or glucose, in the presence of Mn^{2+} was added, increased polarization was observed indicating complex formation between DNS-αlactalbumin and galactosyltransferase. Increased polarization was also observed when UDPgalactose in the presence of Mn^{2+}

was added, again indicating complex formation. The rates of change of fluorescence polarization of fluorescein isothiocyanate labeled human plasma factor XIII were studied as a function of protein concentration and calcium concentration.[28] It was, by polarization measurements, possible to follow the calcium induced dissociation of the tetrameric factor XIIIa, and to observe both a fast and slow phase.

3. Measurement of Rotational Diffusion

When the polarization of a macromolecule-fluorophore conjugate is studied as a function of either temperature or viscosity, it can be seen from Equation 28 that a plot of 1/P vs. T/η will yield a value for the limiting polarization P_o from the intercept of the plot, and, if the fluorescent lifetime is known, the rotational relaxation time ϱ may be calculated from the slope of the plot. Rawitch et al.[29] used this approach to measure the rotational diffusion of thyroglobulin in solution. From temperature dependence studies of the limiting polarization (which was also found to change, indicating changes in microviscosity of the probe environment, as well as changes in measured polarization as the visosity changed), an activation energy for these local environmental changes was calculated. Normal behavior in a 1/P vs. T/η is a linear plot intercepting at $1/P_o$. However, where the system contains more than one rotational relaxation time, or more than one lifetime, the plot will be concave downwards. Such plots can be used to show such deviations from normality, and may be interpreted in terms of either multiple labels, with different lifetimes, or mixtures of molecular species under the conditions studied with different molecular weights or conformations and hence different rotational correlation times.

4. Assignment of Transitions

The limiting polarization is dependent upon the orientation of the absorption dipole and the emission dipole as discussed earlier. Thus polarization measurements can be used to determine whether or not a single transition is responsible for a particular absorption band (excitation band). If a single transition is responsible for excitation, then the observed polarization will be independent of the excitation wavelength.[30] If the absorption band is made up of different transitions however, the polarization vs. excitation wavelength plot will shown a transition between the two absorption transitions.

IV. FLUORESCENT LIFETIMES

A. Introduction

The relaxation of an excited fluorophore to the ground state is an exponential process and follows the law:

$$F_t = F_o e^{-kt} \tag{29}$$

Where F_t and F_o are the fluorescence intensities at times t and zero, respectively, K is the rate constant of decay, and t is the time at which F_t is measured. If we consider the point after excitation at which $F_t = (1/e)\, F_o$ we get:

$$\frac{1}{e} F_o = F_o e^{-kt}$$

$$\therefore \frac{1}{e} = e^{-1} = e^{-kt}$$

$$\therefore 1 = kt$$

$$\therefore k = 1/t$$

Defining this time t (at which $F_t = 1/e\ F_o$) as the fluorescence lifetime (T) it is apparent that $K = 1/T$ so that Equation 29 becomes

$$F_t = F_o e^{-t/\Upsilon} \tag{30}$$

B. Measurement of Fluorescence Lifetimes

There are three principle ways that may be used to experimentally determine T, which differ not only in their experimental design, but also in the range of T they can determine.

1. Exponential Decay Method

This method is based on Equation 30. If it is assumed that an infinitely short light pulse is used to excite a fluorescent species and the emission is monitored as a function of time, a plot of In $[F_o/F_t]$ vs. t will give a straight line of slope 1/T. In practice however, the light pulse will have a finite decay time of its own (which is usually of the order 0.5 to 5×10^{-9} sec), which can be measured using a scattering solution in the absence of the fluorophore, and T pulse can be determined. The measured lifetime in the presence of the fluorophore can now be corrected for the contribution of the light pulse.

$$\Upsilon_{measured} \simeq \Upsilon_{fluor} + \Upsilon_{pulse}$$

The lifetime of the fluorescent species is calculated by using a convolution integral. Obviously the lifetime of the pulse will limit the range of fluorescent lifetimes that can be accurately measured using this approach. In general, lifetimes of the order of 1 to 5 nsec and longer may be determined using this method. The (F_oF_t) vs. t plot used can also give information concerning the homogeneity of the fluorophore. If a single fluorescing species is present a linear plot is obtained. If two or more fluorescing species are present the plot becomes curved. Depending on the separation of the T values, individual T values can be obtained from such a plot. Thus lifetime measurements can be used to indicate purity of the fluorophore.

2. Cross-Correlation Phase Modulation

Consider the case where the frequency of excitation W is comparable to the rate constant K for emission in Equation 29. In this case, the fluorescent species progressively lags behind the exciting light in phase, and becomes attenuated. When $W/K = 1$, the phase of the emitted light is 45° out of phase with the exciting light, and ap-

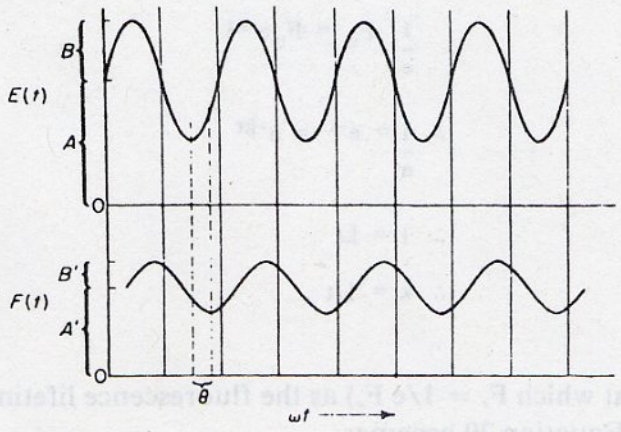


FIGURE 21. Schematic representation of the determination of Θ, the phase lag between excitation and emission. (From Pesce, A. J., Rosen, C. G., and Pasby, T. L., *Flourescence Spectroscopy; an Introduction for Biology and Medicine*, Marcel Dekker, New York, 1971. By courtesy of Marcel Dekker, Inc.)

proaches 90° as the attentuation becomes complete. It has been shown that the excitation of a fluorophore with modulated light of frequency f may be described by Equation 31.[31]

$$E(t) = A + B \cos 2\pi ft \tag{31}$$

Where A > B, the modulation of the exciting light is B/A. A and B are defined by Figure 21. The fluorescence of the population is described by:

$$f(t) = A + B \cos\theta \cos(2\pi ft - \theta) \tag{32}$$

$$\tan\theta = 2\pi ft = w\Upsilon \tag{33}$$

The relative modulation M between the fluorescence and excitation is defined by Equation 34.

$$M = \frac{\text{modulation of fluorescence}}{\text{modulation of excitation}}$$

where the modulation of fluorescence is $(A'-B')/(A+B)$ in Figure 20, and the modulation of excitation is $(A-B)/(A+B)$

$$M = \cos\theta = (1 + 4\pi^2 f^2 \Upsilon^2)^{-1/2}$$
$$= (1 + w^2 \Upsilon^2)^{-1/2} \tag{34}$$

T may thus be determined either by measurement of θ, or by the measurement of M. If a single fluorophore is present, the same value of T will be determined from either Equation 33 or Equation 34. If a mixture of fluorophores is present different T values will be obtained.

3. From Polarization Measurements

As discussed in Section III above, polarization is dependent on the fluorescent lifetime of the fluorophore. If all the parameters in the Perrin equation, Equation 28, are either known, or can be experimentally calculated with the exception of the lifetime, a value for the lifetime can be calculated. However, due to the inherent uncertainties in measuring molecular volumes, and making the assumption that the fluorophore is a sphere, this is not a method of choice. A further drawback is that unlike the above methods, heterogeneity does not lead to an easily distinguishable deviation from the behavior of a single fluorophore.

C. Uses of Lifetime Measurements

Many of the uses of fluorescent lifetime measurements involve one or other of the parameters discussed in this chapter. In combination with polarization measurements, rotational relaxation times can be determined as described earlier. Lifetime measurements are frequently used in conjunction with fluorescence quenching measurements or resonance energy transfer measurements to be described later. Lifetime measurements have been used to describe different fluorophores in a system. For example, using lifetime measurements at different wavelengths, it was found that the fluorescence emission of *Staphylococcus aureus* endonuclease[32] could be resolved into two components, tyrosine emission, with a lifetime of 1.8 nsec and tryptophan emission, with a decay time of 5.5 nsec. It was found that thymidine 3′5′ bisphosphate decreased the intensity of the tyrosine emission and not the tryptophan emission. In combination with anisotropy measurements, the rotational correlation time of the tryptophan fluorescence was found to be 8.9 nsec. This time was not affected by binding of thymidine 3′5′ biphosphate, indicating that no gross structural modifications were induced by this ligand binding. Lifetime measurements of 1-anilino-naphthalene-8-sulfonate (ANS) bound to phosphorylase b[33] indicated that two different classes of ANS binding sites existed with lifetimes of 8 nsec and 18 nsec. Binding of the allosteric activators AMP or IMP, or the conversion of phosphorylase b to phosphorylase a was found to result in a decreased amplitude of the 18 nsec component, rather than the 8 nsec component, most probably as a result of the release of the long lifetime component following a change in conformation. Lifetime measurements in conjunction with anisotropy measurements have also been used to show the interaction of proteins[34] with aspartate aminotransferase and glutamate dehydrogenase. The use of nanosecond fluorescence spectroscopy in biological systems has been extensively reviewed.[35]

V. FLUORESCENCE QUENCHING

A. Theoretical Background

We have discussed in the last section the measurement of the fluorescence lifetime T, and have defined T as equal to 1/K where K is the rate constant for emission. However, there are a number of other processes that can compete with emission as possible routes for the electron in the first singlet excited state returning to the ground state. These were mentioned earlier in reference to Figure 3. We can define a lifetime in the absence of any of these processes T_o as above. The other processes we must consider are internal conversion, whereby the energy of the excited state electron is

eventually taken up by vibrational degrees of freedom in the surrounding solvent. These processes can be designated by a rate constant K_3. Secondly, we must consider intersystem crossing, to which we shall give the rate constant K_2. Intersystem crossing occurs when the excited state electron changes its spin, giving a triplet state. As discussed in Chapter 2, this process has a low probability (K_2 is slow). Deactivation from the triplet state can occur either as a non-radiative process such as internal conversion, which is fast, or by light emission (phosphorescence), which is a relatively slow process since it too is a spin-forbidden transition because on going from the triplet state to the ground state directly spin inversion must occur. Phosphorescence is not normally observed at room temperature, though at low temperatures, where internal conversion processes are slower, phosphorescence can be observed.

The third process to be considered is that of specific collisional quenching. This is the result of collisional processes with an added quencher molecule. Such processes can be described by a second order rate constant, K_5, which shows a dependence on the concentration of the added quencher molecule.

We can define a parameter of the observed fluorescence in the presence of an added quencher molecule, the quantum yield, which as discussed earlier is defined as the ratio of the number of quanta emitted to the number of quanta absorbed. If we assume that the quanta absorbed must be dissipated by one of the above processes,

$$q = \frac{K}{K + K_2 + K_3 + K_5 [Q]} \tag{35}$$

where the rate constants (K) are as defined above, and [Q] is the concentration of added quencher. We can also define an experimental lifetime for the excited state, T, which is a measure of all of the above deactivation processes that are occurring:

$$\tau = \frac{1}{K + K_2 + K_3 + K_5 [Q]} \tag{36}$$

Since in the absence of added quencher the lifetime, T_o can be defined as

$$\tau_o = \frac{1}{K + K_2 + K_3} \tag{36A}$$

we can derive an expression for the quantum yield q in the presence of added quencher in terms of T and T_o:

$$q = \frac{\tau}{\tau_o}$$

In the absence of the added quencher we can define the quantum yield q_o as:

$$q_o = \frac{K}{K + K_2 + K_3} \tag{37}$$

The ratio of the quantum yields in the absence and the presence of the added quencher can be obtained from Equation 35 and Equation 37:

$$\frac{q_o}{q} = \frac{K + K_2 + K_3 + K_5 [Q]}{K + K_2 + K_3} \tag{38}$$

Using the expression for the lifetime measured in the absence of added quencher, Equation 36A we get:

$$\frac{q_o}{q} = 1 + K_5 [Q]\gamma \tag{39}$$

When the excitation intensity and the concentration of the fluorophore are kept constant, the measured fluorescence intensity is proportional to the quantum yield. Hence:

$$\frac{F_o}{F} = 1 + K_5 [Q]\gamma \tag{40}$$

Where f_o is the fluorescence intensity in the absence of added quencher, and f is the fluorescence intensity in the presence of added quencher. This equation is the Stern-Volmer equation. For quenching that is due to collision with the excited state, it is apparent that the measured lifetime T of the fluorophore is decreased by addition of the quencher.

A second type of quenching of fluorescence can also occur, which involves the formation of a complex between the quencher and the ground state of the absorbing molecule, which either has no fluorescence ($q = 0$) or has a fluorescence with a very small quantum yield. An expression very similar to the Stern-Volmer equation above (Equation 40) can be derived for this situation:

$$\frac{F_o}{F} = 1 + K_A [Q] \tag{41}$$

$$K_A = \frac{[\text{complex}]}{[A]\,[Q]}$$

where $K_A = [\text{complex}]/[A]\,[Q]$ with [A] being the concentration of the ground state. This type of quenching however, does not affect the lifetime of the excited state. These two mechanisms of quenching can be distinguished on the basis of the lifetime dependence on quencher concentration.

A modified Stern-Volmer equation can be derived[36] for situations where both collisional quenching and so called "dark complex" or static quenching occur:

$$\frac{F_o}{F} = (1 + K_s [Q] \tau) (1 + K_A [Q]$$

The Stern-Volmer equation, Equation 40 predicts a linear relationship between F_o/F and the concentration of a collisional quencher. Downward curvature of a Stern-Volmer plot may be explained by two populations of tryptophans, one of which is inaccessible to the quencher. Lehrer[37] has derived an equation, Equation 42.

$$\frac{F_o}{\Delta F} = \frac{1}{[Q]} f_a k_q + 1/f_a \qquad (42)$$

Where ΔF is the change in fluorescence due to the addition of a given quencher concentration, [Q], K_Q is the quenching constant of the accessible tryptophans ($= K_s \gamma_o$) and f_a is the fraction of tryptophans which are accessible. A plot of $F_o/\Delta F$ vs. [Q] intercepts at $1/f_a$, allowing the fraction of accessible tryptophan residues to be experimentally determined.

B. Uses of Quenching Measurements

A variety of quenching molecules have been added to systems to measure quenching of protein fluorophores. Quenching by such added quenchers has been used to assess the accessibility of the fluorophore to the quenching molecule. Results from such experiments have been used to assess the environment of the fluorophores, and changes in the environment as a result of conformational changes. Lehrer[37] has used a series of model compounds with different charges and quenching of their fluorescence by iodide ions to assess the state of the tryptophyl residues in lysozyme. With native lysozyme a linear dependence of quenching on iodide concentration was observed over iodide concentrations of 0 to 0.2 *M*. The lack of curvature suggests that the fluorescence of only one class of tryptophans was affected. In the presence of guanidinium hydrochloride all of the tryptophan residues were quenched, again giving a linear response to iodide concentration. It may be noted that this selective quenching of only one class of tryptophans in lysozyme indicates that in this case there is little energy transfer from buried to exposed tryptophans. If there had been such energy transfer, uniform quenching of all the tryptophans would be observed. The observation that the quenching by iodide increased as pH decreased indicating most probably the loss of negative charge of the protein, probably due to protonation of carboxylate groups of glutamate or aspartate, near to the tryptophan residues.

A variety of other quenching molecules have been used to monitor accessibility of a fluorophore to the quencher, including uncharged molecules such as acrylamide, which is fairly polar,[38] and trichloroethanol,[39] which is also uncharged, but is much less polar. When acrylamide is used with single tryptophan-containing proteins, Figure 22, it is found that the Stern-Volmer equation is obeyed. With multiple tryptophan-containing proteins such as lysozyme and α-chymotrypsin, Figure 23, a marked curvature downwards is observed as the acrylamide concentration increases, indicating the existence of more than one class of tryptophan residues. The results with lysozyme obtained using acrylamide are very similar to those obtained with iodide discussed above. Results of acrylamide quenching with other multiple tryptophan-containing proteins are shown in Figure 24. Although the plots are linear with the native proteins, a marked curvature upwards is observed with each of the denatured proteins. This curvature in

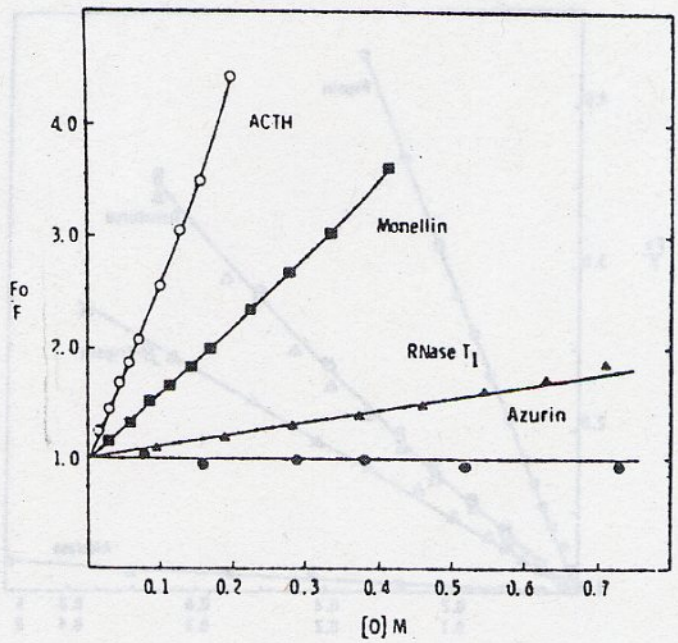


FIGURE 22. Acrylamide quenching of single tryptophan containing proteins. (Reprinted with permission from Eftink, M. R. and Ghiron, C. A., *Biochemistry*, 15, 672, 1976. Copyright by the American Chemical Society.)

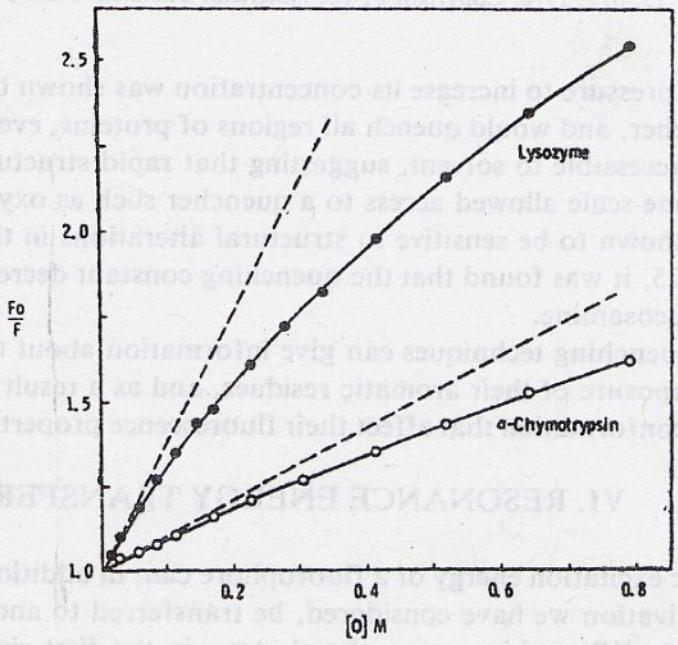


FIGURE 23. Selective quenching of lysozyme or chymotrypsin fluorescence by acrylamide. The dashed lines indicate the initial slopes. (Reprinted with permission from Eftink, M. R. and Ghiron, C. A., *Biochemistry*, 15, 672, 1976. Copyright by the American Chemical Society.)

a Stern-Volmer plot indicates that static quenching, as well as collisional quenching is occurring.

Another quenching molecule that has been introduced and has some potential in observing accessibility and structural changes that affect accessibility, is oxygen.[40,41]

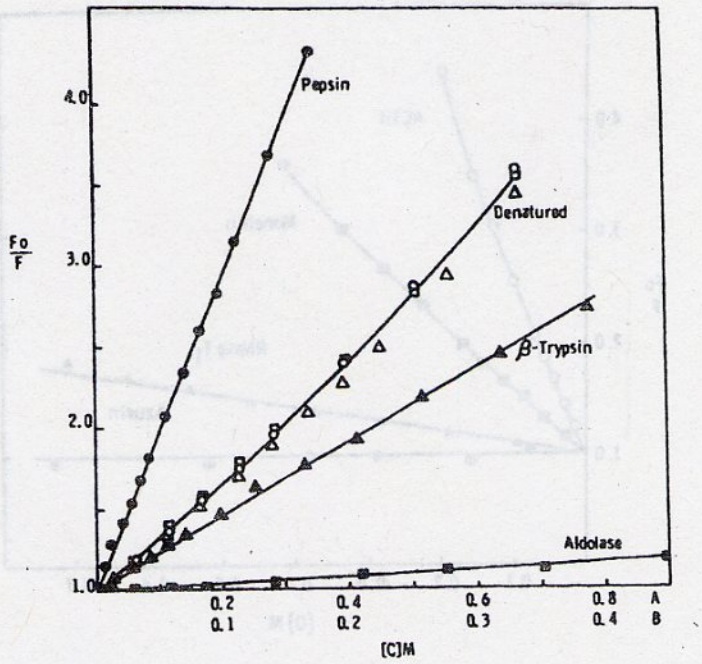


FIGURE 24. Acrylamide quenching of multiple tryptophan containing proteins. The open symbols (scale B) are for the proteins denatured in 6.7 *M* Gdn. HCl. (Reprinted with permission from Eftink, M. R. and Ghiron, C. A., *Biochemistry*, 15, 672, 1976. Copyright by the American Chemical Society.)

Oxygen, at high pressure to increase its concentration was shown to be a very efficient collisional quencher, and would quench all regions of proteins, even those usually considered to be inaccessible to solvent, suggesting that rapid structural fluctuations, on a nanosecond time scale allowed access to a quencher such as oxygen. The quenching by oxygen was shown to be sensitive to structural alterations in the protein. With lysozyme, Figure 25, it was found that the quenching constant decreased in the presence of di-*N*-acetylglucosamine.

All of these quenching techniques can give information about the structure of proteins, and the exposure of their aromatic residues, and as a result can be used to indicate changes in conformation that affect their fluorescence properties.

VI. RESONANCE ENERGY TRANSFER

The electronic excitation energy of a fluorophore can, in addition to the other mechanisms of deactivation we have considered, be transferred to another, well-separated molecular system. When this occurs, the electron in the first singlet excited state of the fluorophore (Donor) returns to the ground state, and an electron in the second molecule (Acceptor) is excited to the singlet excited state. Such long range singlet-singlet energy transfer requires a dipole-dipole resonance interaction between the donor and the acceptor pair.[42] The interaction energy between the two dipoles depends on $1/R^3$, where R is the distance between the donor and the acceptor. The rate of energy transfer ($K_D \rightarrow A$) is proportional to the square of the interaction energy. As a result, $K_D \rightarrow A$ is proportional to $1/R$.[6]

We can define a distance, R_o, where the rate of transfer is equal to the rate of emission. This distance, R_o, the so-called critical distance of energy transfer, has been shown by Forster[42] to be:

$$(R_o)^6 = \frac{\text{Constant} \cdot K^2 \cdot C \cdot \tau_o}{16\,\pi^4\,\eta^2\,N^2\,\bar{\nu}_o - 2} \qquad (43)$$

Where K is an orientation factor, τ_o is the fluorescence lifetime of the donor in the absence of resonance energy transfer, η is the refractive index at the wavelength of emission, $\bar{\nu}_o$ is the wavenumber of the o-o transition, and J is the so-called overlap integral. The overlap integral can be regarded as a measure of the strength of the dipole-dipole resonance interaction between the donor and the acceptor. This integral can be calculated from the overlap of the donor fluorescence emission spectrum, and the acceptor absorbance spectrum. Calculation by this means has been described by Weber.[43]

The rate of energy transfer in a particular system is:

$$K_{D \rightarrow A} = \frac{1}{\tau_s}\left(\frac{R_o}{R}\right)^6 \qquad (44)$$

The quantum yield of the donor in the absence of resonance energy, q_o is again given by the expression used earlier.

$$q_o = \frac{K}{K + \Sigma K_s} \qquad (45)$$

In the presence of resonance energy transfer an additional mode of energy dissipation must be incorporated into Equation 45 to give the quantum yield of the donor in the presence of resonance energy transfer, q_{ret}.

$$q_{ret} = \frac{K}{K + \Sigma K's + K_{D \rightarrow A}} \qquad (46)$$

Using an expression for $\tau_s = 1/K \rightarrow \Sigma K's$, we get:

$$q_{ret} = q_o \frac{1/\tau s}{1/\tau s + K_{D \rightarrow A}} \qquad (47)$$

Substituting the equation (Equation 44) for $K_{D \rightarrow A}$ into Equation 47 we get Equation 48.

$$q_{ret} = q_o \frac{1}{1 + (R_o/R)^6} \qquad (48)$$

So far we have discussed the effects of an acceptor molecule on the fluorescence quan-

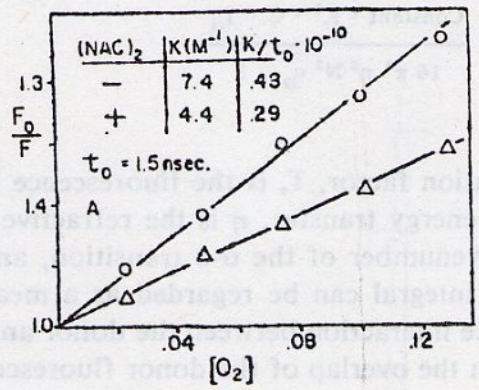


FIGURE 25. Oxygen quenching of lysozyme fluorescence in the presence (Δ) and absence (o) of di-*N*-acetylglucosamine. (Reprinted with permission from Lakowicz, J. R. and Weber, G., *Biochemistry*, 12, 4161, 1973. Copyright by the American Chemical Society.)

tum yield; however if the acceptor molecule, as well as absorbing energy can also emit fluorescence, we can determine energy transfer by means of the sensitized fluorescence of the acceptor.

From Equation 48 we get:

$$\frac{q_{ret}}{q_o} = \frac{1}{1 + (R_o/R)^6} \tag{49}$$

Defining an efficiency of energy transfer, E,

$$E = 1 - \frac{q_{ret}}{q_o} \tag{50}$$

we can see that:

$$E = 1 + \frac{1}{1 + (R_o/R)^6} = \frac{(R_o/R)^6}{1 + (R_o/R)^6} \tag{51}$$

From Equation 51 we get:

$$R = R_o\ (E^{-1} - 1)^{1/6} \tag{52}$$

Thus by measuring the quantum yield of the fluorophore in the presence of an acceptor of resonance energy transfer, $q_{ret.}$, and the quantum yield in the absence of an energy transfer acceptor, q_o, the distance between the donor and acceptor can be calculated.

From the above derivation, it is apparent that the efficiency of energy transfer, E, can also be obtained from the lifetimes in the presence or absence of resonance energy transfer,

$$E = 1 - \Upsilon_{ret}/\Upsilon_o \tag{53}$$

Where T_{ret} is the lifetime of the fluorophore in the presence of an acceptor for resonance energy transfer, and T_o is the lifetime of the fluorophore in the absence of an acceptor.

Since the quantum yield of the fluorophore is directly proportional to the fluorescence intensity at a given wavelength, we can also define the efficiency of transfer by Equation 54.

$$E = 1 - \frac{F_{ret}}{F_o} \tag{54}$$

Where F_{ret} is the fluorescence intensity in the presence of the acceptor of energy transfer, and F_o is the fluorescence intensity in the absence of the acceptor of energy transfer.

On the face of it, resonance energy transfer measurements and calculations might seem to be simple. However, several warning points must be examined. Perhaps the severest is that the equations derived above apply only if there is a single class of donor and a single class of acceptor. Since in many instances resonance energy transfer measurements utilize covalently bound fluorophores and sometimes acceptors as well, it is imperative to establish uniqueness of labeling.

The other problems that must be dealt with involve the various terms in Equation 43 by which the critical distance R_o is calculated. The critical distance R_o is specific for a particular pair of donor and acceptor molecules. The overlap integral J is estimated from the equation:

$$\mathbf{J} = \int_o^\infty \epsilon A\,(\bar{\nu})\,\epsilon D\,(2\bar{\nu}_o - \bar{\nu})\,d\bar{\nu} \tag{55}$$

Where $\varepsilon A\,(\bar{\nu})$ is the molar decadic extinction coefficient of the acceptor at wave-number $\bar{\nu}$, and $\varepsilon_D\,(2\bar{\nu}_o - \bar{\nu})$ is that of the donor at wavenumber $2\bar{\nu}_o - \bar{\nu}$.[2,44]

The term that receives the most attention is K, which is an orientation factor that depends on the angle between the oscillators of the donor and the acceptor. It is usually assumed that if both oscillators are undergoing rapid rotation, then $K^2 = {}^2/_3$.[44] the range of possible values for K^2 is 0 to 4.0. When R_o is calculated for values of K^2 from 0.1 to 4.0 for the dansyl → cobalt system, R_o varies from 14.6 Å to 27 Å; the value using $K^2 = {}^2/_3$ is 23 Å.[45] As can be seen, a change in the orientation factor upon binding of a subsequent ligand could lead to interpretations of distance changes upon ligand binding rather than orientation changes. If both donor and acceptor are fluorescent, polarization measurement can give information about the mobility of each, and hence the reasonableness of the $K^2 = {}^2/_3$ assumption. In terms of distance mea-

surements, reasonable limits on the confidence of a "distance measurement" can be calculated using several values for K^2.

Two other variables in Equation 43 should also be discussed η, the refractive index of the medium, which in protein systems is assumed to be 1.33,[46] and T_o, the fluorescence lifetime of the donor in the absence of resonance energy transfer. From the form of Equation 43, it is easy to see that even quite large changes in η values will have very little effect on the calculation of R_o. It has also been shown,[45] that quite large changes in T_o will also cause rather small changes in R_o values. The expression for R_o has also been derived using the quantum yield of the donor (Q_o) in the absence of resonance energy transfer, which is of course related to the lifetime T_o; this expression is given below, Equation 56.[47]

$$R_o = \text{Constant } (J \cdot K^2 \cdot Q_o \cdot \eta^{-4})^{1/6} \qquad (56)$$

Thus, reasonable limits can usually be set on distance measurements using resonance energy transfer measurements. Care must, however, be taken in the interpretation of changes in distances measured using resonance energy transfer as these can be due to changes in orientation of donor and acceptor rather than changes in distance. In all distance measurements using resonance energy transfer, it is a good idea to base conclusions on as many different measurements as possible — using lifetime measurements as well as quantum yield measurement where possible, and, if possible using several fluorescent probes to measure the same distance. In this way added confidence may be placed in the results.

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