Preliminary Notes

On the nature of the fluorescence of enzyme–DPNH complexes

The discovery by BOYER AND THEORELL[1] that the addition of a DPNH-linked enzyme to a solution of DPNH causes an increase in the intensity of fluorescence and a shift of the emission spectrum to shorter wavelengths, has provided an elegant and versatile tool for the investigation of enzyme–coenzyme complexes. However, the usefulness of such studies has been sharply limited by the lack of any generally valid and experimentally substantiated explanation of the phenomenon. Hypotheses involving changes in the structure, conformation, planarity, or electron distribution of the reduced nicotinamide ring have been widely proposed.

We have found, however, that the binding of DPNH in *any* manner which reduces the possibility of inter- or intra-molecular collisions will cause both an increase in fluorescence intensity and a shift of the emission spectrum to shorter wavelengths.

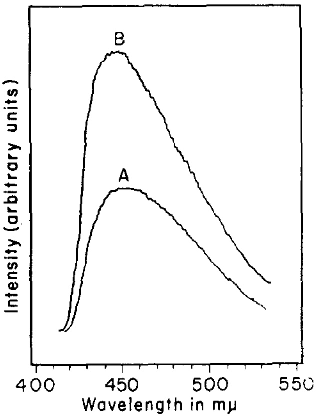

Fig. 1. The fluorescence emission spectra of DPNH and of an insoluble complex of DPNH, Zn^{++}, and phosphate. Curve A, $2 \cdot 10^{-5}$ *M* DPNH in 0.2 *M* Tris buffer, pH 7.6. Curve B, same solution after addition of $ZnSO_4$ to a final concentration of $7.5 \cdot 10^{-3}$ *M*, and sodium phosphate to a final concentration of $1.8 \cdot 10^{-3}$ *M*. Spectra were recorded with a Farrand spectrofluorometer equipped with a 1 P 21 photomultiplier tube and an Osram XBO 162 lamp, using 10-mµ slits. A quartz cuvette (1 cm × 1 cm) was used with a Corning 7–54 filter in front and a 3–72 filter behind it. The activation wavelength was 345 mµ. The fluorescence of all components other than DPNH was negligible.

Both phenomena are demonstrated in Fig. 1. The addition of zinc and phosphate ions to a solution of DPNH causes the formation of an insoluble complex. The stirred suspension shows an increase in fluorescence intensity of 92 %. Since the spectra are uncorrected for changes with wavelength of lamp emission, photomultiplier-tube sensitivity, and transmittance of filters, the exact shape of the curves has only relative meaning; however, the spectral shift has the same magnitude and direction as that caused by the addition of L-glutamic dehydrogenase to a solution of DPNH under the same instrumental conditions. These phenomena are not caused by scattered light. The identical shape of the activation spectra in the presence of and in the absence of the complex, and the unchanged fluorescence of a DPNH solution in the presence of stirred suspensions of sea sand or silica gel, prove that the optical-filter system is effectively preventing any scattered light from reaching the photomultiplier tube.

Abbreviations: DPNH, reduced diphosphopyridine nucleotide; Tris, tris(hydroxymethyl)-aminomethane.

The relative increase in fluorescence intensity when complexes are formed between DPNH and a variety of other substances are listed in Table I. It can be seen that an increase in fluorescence intensity occurs whenever the reduced coenzyme is either incorporated into a solid phase by complex formation, or is bound to the surface of a large molecule already present in suspension.

TABLE I

INCREASE IN DPNH FLUORESCENCE INTENSITY IN VARIOUS COMPLEXES

Substances added to DPNH solution	*Presence of a precipitate*	*Increase in fluorescence intensity (%)*
Zn^{++}, phosphate	+	50
Zn^{++}, phosphate, albumin	+	86
Silica gel	+	0
Mg^{++}	—	0
Mn^{++}	+	102
Dowex 1-X-4	+	34

Experimental procedures and instrumental conditions were the same as for Fig. 1. DPNH concentration was $2 \cdot 10^{-5}$ *M* in all experiments. Final concentrations of added components were: phosphate, $1.8 \cdot 10^{-3}$ *M*; Zn^{++}, Mg^{++}, and Mn^{++}, $7.5 \cdot 10^{-3}$ *M*; bovine serum albumin, 2.7 mg/ml. Fluorescence emission intensities were read at 465 mμ.

It is well established that in any group of organic molecules, fluorescence is strongest in those compounds which contain the most rigid nuclear framework, or which are, in some manner, protected from deactivating collisions[2]. The increased intensity of fluorescence of dyes adsorbed on colloids was first noticed in 1895 by WIEDEMANN AND SCHMIDT[3]. DPNH is a particularly flexible molecule possessing many degrees of freedom. Such a molecule, having reached an excited state by absorption of a quantum of light, may dissipate that energy by any of a number of competing pathways, of which fluorescent emission is only one. Of these competing pathways, only two concern us here; namely, internal conversion, and solvent quenching. If a highly flexible molecule is bound by more than one point of attachment to some relatively inflexible surface, the possibilities for internal conversion are greatly reduced, and the number of deactivating collisions with solvent molecules may be lessened.

Thus it can be seen that an increase in intensity and a shift to shorter wave lengths of the fluorescence of a reduced coenzyme caused by the addition of an enzyme, is to be interpreted as evidence *only* of the binding of the reduced coenzyme on the surface of the enzyme.

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