oxidation of chlorophyll[5,6] and bacteriochlorophyll.[44] Considering MgTPP or MgOEP as model systems, we suggest that the oxidized chlorophylls are properly characterized as $\pi$-cation radicals. Indeed, electro-oxidation[1] of ethyl chlorophyllide a and chlorophyll a converts these molecules into bleached species whose optical and esr properties are consistent with this identification. An additional feature of the magnesium porphyrins is that these molecules are easily oxidized, a fact which may account for the appearance of magnesium chlorins in the photosynthetic apparatus. In the instance of catalase and peroxidase function, the oxidation of the prosthetic heme group is usually formulated as metal oxidation[4] to yield Fe(IV) and Fe(V). An alternative, tentatively supported by our results, suggests that ring oxidation as well as metal oxidation should be considered.

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**Acknowledgments.** We wish to thank Professor D. Mauzerall for discussions on the nature of the oxidation products and D. van der Kolk for technical assistance. This work was supported in part by the U. S. Atomic Energy Commission, in part by the Petroleum Research Fund of the American Chemical Society (PRF-849-Gl), and in part by the Research Corporation.

# Deuterium Isotope Effects on the Fluorescence of Tryptophan in Peptides and in Lysozyme[1]

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**Abstract:** A study has been made of the variation of fluorescence with pH and pD for the tryptophan model compounds, skatole, L-trp, L-trp-L-tyr, and N-Cbz-L-trp-L-tyr, and for the tryptophyl fluorescence of lysozyme and of the lysozyme–triNAG complex. Shifts of the fluorescence transitions by 0.3–0.7 pH unit in $D_2O$ compared to $H_2O$ gave further evidence for the involvement of amino, carboxyl, and phenol groups in the quenching of tryptophyl fluorescence. The large isotope effects observed are consistent with the expected decreased rate of deuteron transfer when the protons of the amino and carboxyl groups are exchanged for deuterons in $D_2O$. The value for the ratio of the rate constant for the intramolecular quenching by the $NH_3^+$ group of L-trp in $H_2O$ and $D_2O$ was calculated to be 2.7 from the experimental data and a simple kinetic scheme. A large isotope effect was also observed for the intermolecular quenching of indole-3-acetamide by glycine at neutral pH. These data are consistent with a proton transfer quenching mechanism. The results of the study with lysozyme provide further evidence for the involvement of acid groups in the quenching of tryptophan fluorescence.

A number of recent studies have dealt with deuterium isotope effects on the fluorescence of indole and tryptophan compounds.[2-4] Stryer, for example, showed that for a variety of fluorophors with exchangeable protons the slower rates of deuteron transfer *from the fluorophor* to the solvent adequately explained the isotope effect.[2] The possibility appears, however, that deuterium isotope effects can also be caused by the decreased rate of proton transfer from a quencher *to the fluorophor*. This will be especially important for those systems in which the protonated form of the excited fluorophor has reduced fluorescence, as appears to be the case for indole compounds.[5] In particular, intramolecular proton transfer from the amino and carboxyl groups to the indole ring of L-trp[6] and tryptophyl dipeptides has been suggested as a quenching mechanism.[7] In view of the slower transfer rate of the deuteron, fluorescence differences are to be expected in these systems. In proteins such effects may be observable if a proton donating group is located near an indole side chain. This study of tryptophyl fluorescence as a function of pH in $H_2O$ and $D_2O$ provides further evidence that a major contribution to the deuterium isotope effect observed in such compounds is due to proton transfer to the fluorophor.

## Experimental Section

Highly purified samples of L-trp (Mann Research, N. Y.), L-trp-L-tyr (Cyclo Chemical Corp., Los Angeles, Calif.), and N-Cbz-L-trp-L-tyr (Cyclo) were used without further purification. Lysozyme was obtained from Worthington Biochemical Corp., Freehold, N. J., and triNAG was obtained through the courtesy of Dr. J. Rupley. Indole (Fisher Scientific, Boston) and skatole

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(6) The following abbreviations are used: L-trp = L-tryptophan; L-tyr = L-tyrosine; N-Cbz = N-carbobenzoxy; triNAG = tri-N-acetyl-D-glucosamine.

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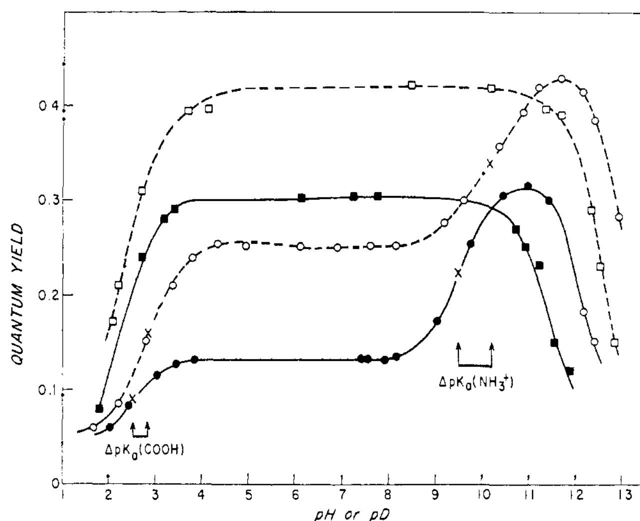


Figure 1. Fluorescence variation with pH and pD of skatole and L-trp: L-trp, in $H_2O$ (-●-●-); in $D_2O$ (-○-○-); skatole, in $H_2O$ (-■-■-); in $D_2O$ (-□-□-). Crosses indicate the estimated $pK_a$ associated with the fluorescence transition; concentration $1.8 \times 10^{-5}$ $M$ in 5 m$M$ HEPES buffer, in $H_2O$ or 99.8% $D_2O$, excitation at 280 nm.

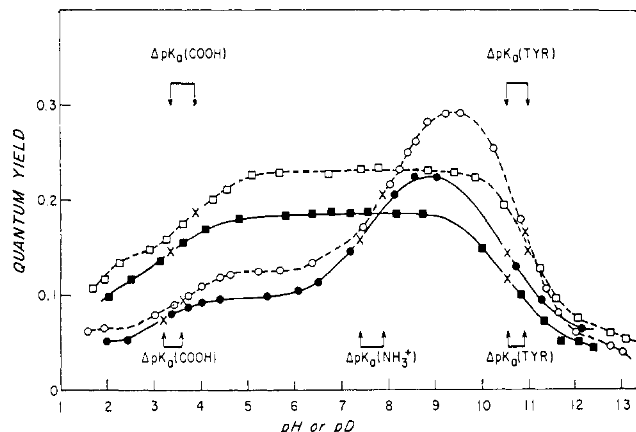


Figure 2. Fluorescence variation with pH and pD of L-trp-L-tyr and N-Cbz₂-L-trp-L-tyr: L-trp-L-tyr, in $H_2O$ (-●-●-); in $D_2O$ (-○-○-); N-Cbz-L-trp-L-tyr, in $H_2O$ (-■-■-); in $D_2O$ (-□-□-). Smaller symbols indicate the estimated $pK_a$ associated with the fluorescence transition; concentration $1.8 \times 10^{-5}$ $M$ in 5 m$M$ HEPES buffer, in $H_2O$ or 99.8% $D_2O$; excitation at 280 nm.

(Mann) were recrystallized from hot water containing Norit A (Matheson Coleman and Bell, Rutherford, N. J.).

Stock solutions of the model compounds were made by dissolving a few crystals in deionized distilled $H_2O$ or $D_2O$ (Merck, Rahway, N. J.) 99.8% pure which contained 5 m$M$ N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (Calbiochem, Los Angeles, Calif.) at neutral pH. The solutions were filtered through a Millipore filter (HAWP 0.45 $\mu$) and diluted so that $A_{280}$ (1 cm) $\approx$ 0.1 (measured in 4-cm cuvets). Titrations were performed in two steps on different samples with 0.5 $M$ HCl or DCl (Merck) for the neutral–acid pH range and with 0.5 $M$ NaOH or NaOD (Merck) for the neutral–basic pH range. The titrant was added by means of a Digipet micropipet (Manostat Corp., N. Y.). Reversal of fluorescence was checked in each case by the addition of sufficient acid or base to bring the pH back to neutral. Solutions of lysozyme were similarly treated except that titrations covering the entire pH range were run from pH 11 to 2. The amount of triNAG present was sufficient to saturate lysozyme over the entire pH region so that all measurements are for the fluorescence properties of the complex.[8] In order to correct for any fluctuations of the intensity of the exciting radiation, the fluorescence of a standard L-trp solution in $H_2O$ was measured with each sample.

Fluorescence measurements were made with an Aminco-Bowman spectrofluorometer with a band width of approximately 12 m$\mu$ (a few measurements were made with an instrument that employed two Jarrell-Ash monochrometers, an EMI 9601B photomultipler, and an Osram HBO 200 high-pressure 200-W mercury lamp). Quantum yields were calculated by comparing the peak fluorescence intensity of the samples of known absorbance with the fluorescence of L-trp (quantum yield = 0.13).[9] The maximum error in quantum yield is estimated to be about 10% due to the slight differences in spectral band width among the samples and slight changes in spectral band width associated with the ionization of certain groups. Self-absorption corrections were not made due to the small degree of spectral overlap between fluorescence and absorption for tryptophan in aqueous solutions. Corrections were made for the apparent loss of tryptophan fluorescence caused by tyrosine absorption for the dipeptides. No tyrosine fluorescence was detected in the case of the dipeptides. No precautions were taken to remove dissolved oxygen from the solutions because when oxygen-free nitrogen was bubbled through a solution of skatole for a few minutes no change in the fluorescence was measured. It is assumed that all the protons bound to oxygen or nitrogen will exchange readily with $D_2O$ when exposed to this solvent.[10] In the case of lysozyme, the titration was begun at pH 11 where most acid groups are dissociated. Recent nmr studies on lysozyme indicate that all the indole imino protons would also be readily exchangeable under the experimental conditions used here;[11] *i.e.*, most protons that affect tryptophan fluorescence are exchanged for deuterons over the entire pD range. Measurements of pH were made with a Radiometer PHM 4 instrument utilizing a Sargeant combination microelectrode over the entire pH range or an IL combination microelectrode for the acid range. The pD values were calculated by adding 0.4 to the apparent pH.[12] This correction was verified by comparing the pH of an acetate buffer in $H_2O$ and $D_2O$ with the known dissociation constant of acetic acid in $H_2O$ and $D_2O$.[12] All measurements were made at 25 ± 1°.

## Results

The results of the fluorescence titrations in $H_2O$ and $D_2O$ for the tryptophyl model compounds are shown in Figures 1 and 2. In agreement with earlier studies in $H_2O$,[13] the increase of fluorescence in the pH 8–11 region for L-trp and in the pH 6–9 region for the dipeptides is associated with the ionization of amino groups, and the region in fluorescence in the pH 2–5 region is associated with the ionization of carboxyl groups. Apparent $pK_a$'s estimated as the value of pH or pD corresponding to the midpoint of the fluorescence transition are indicated in the figures.

The titrations in $D_2O$ indicated that shifts in $pK_a$'s of 0.3–0.7 unit were caused by the exchange of deuterons for protons. Shifts in $pK_a$ of this magnitude have been measured potentiometrically for various acid groups.[14] The observed quenching for skatole below pH 3 and above pH 10.5 is probably due to direct protonation of the indole ring and to imino proton transfer to $OH^-$, respectively.[7,13] These pH values are shifted for L-trp to below pH 2 and above pH 11,[6] probably because of electrostatic effects. The additional fluorescence loss between pH 9.0 and 12.0 in $H_2O$ and 9.5 and 12.5 in $D_2O$ observed for L-trp-L-tyr and N-Cbz-L-trp-L-tyr (Figure 2) parallels the ionization of tyrosine and may be attributed to the quenching of tryptophan fluores-

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cence by energy transfer.[15] The fluorescence of N-Cbz-L-trp-L-tyr (Figure 2) shows the effect of ionization of only tyrosyl and carboxyl since the amino group is blocked and does not undergo ionization.

There is a small pH range associated with the maximum fluorescence of the indole fluorophor in L-trp. For L-trp this pH range is 10.6–11.2 in $H_2O$ and 11.4–12.0 in $D_2O$. The similar quantum yields of the model compound, skatole, and L-trp in this pH range indicate that the fluorescence is characteristic of the unquenched substituted indole side chains. Good agreement is also obtained for the deuterium isotope effect on fluorescence for skatole and L-trp in the pH range of maximum fluorescence ($Q^D/Q^H = 1.40$ for skatole and 1.37 for L-trp), but the deuterium isotope effect for the zwitterion form of L-trp (plateau value) is greater ($Q^D/Q^H = 1.92$). This latter value can be compared with the previously determined values of 1.65[2] and 2.15[4] for L-trp in water. The larger value observed at neutral pH is apparently due to the greater rate of quenching associated with proton transfer as compared to deuteron transfer from the amino group, as will be discussed in the next section. The smaller isotope effects observed for the fluorescence of the dipeptides result from the lower initial quantum yields due to quenching effects of peptide groups.[16,17]

The intermolecular quenching of indole-3-acetamide by glycine at pH 7.5 in $H_2O$ and $D_2O$ was also studied. The quenching by glycine in $H_2O$ followed a Stern–Volmer dependence (eq 6) with $K_Q = k_3\tau_f = 0.6\ M^{-1}$. The quenching constant in $D_2O$ was 0.1–0.2 $M^{-1}$. This yields $k_3^H/k_3^D = 4$–8 using a value of 1.3 for the fluorescence lifetime ratio.

The fluorescence dependence on pH of lysozyme and of the lysozyme–triNAG complex has been studied in $H_2O$.[18] Since the fluorescence of the lysozyme–triNAG complex was shown to be markedly affected by the ionization of certain acid groups, a similar study in $D_2O$ was done. The results are shown in Figure 3. The shifts in $pK_a$ of these quenching groups in $D_2O$ are further evidence for specific quenching rather than a generalized pH dependent conformational change. Small isotope effects were also obtained for other proteins.[4]

The wavelength of maximum fluorescence in the case of L-trp was observed to be dependent upon the ionization of the amino acid in agreement with other workers[5,17,19] but not upon the solvent ($H_2O$ or $D_2O$). The largest change was a shift from 354 to 348 nm for L-trp between the peak and plateau ranges of the fluorescence variation with pH. A somewhat smaller shift was also observed to be associated with the ionization of the carboxyl group. The wavelength of maximum fluorescence for skatole in $H_2O$ and $D_2O$ was 362 nm and did not vary with pH.

## Discussion

From the results presented above it is clear that another quenching mechanism operates on the fluorescence of amino acids, peptides, and proteins in addi-

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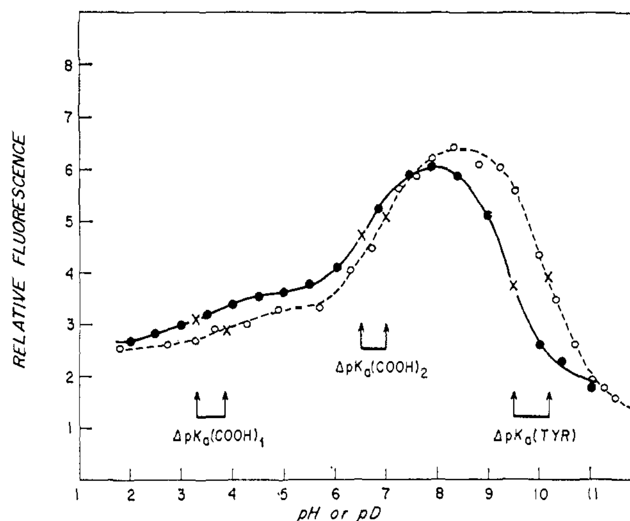
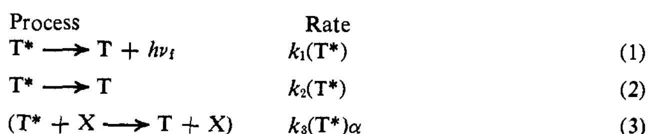



Figure 3. Fluorescence variation of the peak fluorescence of lysozyme–triNAG with pH and pD: in $H_2O$ (–●–●–); in $D_2O$ (–○–○–). Smaller symbols indicate the estimated $pK_a$ associated with the fluorescence transition; lysozyme concentration 0.05 mg/ml; triNAG concentration 0.6 mg/ml, in 0.2 *M* NaCl, 5 m*M* HEPES buffer, in $H_2O$ or 99.8% $D_2O$; excitation at 280 nm.

tion to that observed in indole- and methyl-substituted indoles. Although a larger isotope effect was observed for L-trp than for indole compounds by Stryer[2] and Eisinger and Navon,[4] the possibility of intramolecular quenching by proton transfer to the fluorophor was not considered. This additional quenching process must be included in the explanation of the temperature dependence of the fluorescence of L-trp obtained by Eisinger and Navon.[4] The process occurring between 175 and 225°K, which causes a spectral shift to longer wavelength with little change in quantum yield and no isotope effect, has been explained as an excited state interaction with solvent at temperatures where solvent reorientation can occur.[4] The quenching process which occurs between 225°K and room temperature with a large isotope effect and without spectral shift is postulated, as discussed below, to have a large contribution from activated quenching of the solvated excited state by proton transfer from the amino group. Another process which causes fluorescence quenching of simple indole compounds becomes important above room temperature.[3,4]

Evidence for the involvement of proton transfer can be obtained by the consideration of a simple kinetic scheme for the case of L-trp. To calculate the isotope effect on the second-order rate constant, the following processes and rates can be associated with the deactivation of an excited tryptophan molecule, T*.



| Process | Rate | |
|---|---|---|
| $T^* \longrightarrow T + h\nu_f$ | $k_1(T^*)$ | (1) |
| $T^* \longrightarrow T$ | $k_2(T^*)$ | (2) |
| $(T^* + X \longrightarrow T + X)$ | $k_3(T^*)\alpha$ | (3) |

The first process leads to fluorescence at frequency $\nu_f$ with an intrinsic rate constant $k_1$. It can be assumed that deuteration does not affect this rate since measurements on indole compounds have shown that quantum yields in $H_2O$ and $D_2O$ are proportional to lifetimes[2,3] (*i.e.*, $Q^D/Q^H = \tau_f^D/\tau_f^H$). The second process, which includes radiationless deactivation by solvent interaction,

is isotope dependent since $Q^D/Q^H = 1.3$–$1.4$ for indole compounds and unquenched L-trp (Figure 1).[2,3,6] The third process involves intramolecular quenching by the acid form of the amino group of L-trp ($NH_3^+ = X$), and the fraction of molecules carrying $NH_3^+$ is $\alpha$. The effect of isotope substitution on this quenching process can arise from differences in the transfer rate of $D^+$ and $H^+$ from $-ND_3^+$ and $-NH_3^+$, respectively. In the absence of quencher (un-ionized amino group)

$$Q_0 = \frac{k_1}{k_1 + k_2} = k_1\tau_f \quad (4)$$

where $\tau_f = 1/(k_1 + k_2)$; in the presence of quencher (ionized amino group)

$$Q = \frac{k_1}{k_1 + k_2 + k_3\alpha} \quad (5)$$

Combination of eq 4 and 5 results in

$$\frac{Q_0}{Q} - 1 = k_3\tau_f\alpha \quad (6)$$

The deuterium isotope effect can be expressed as an effect on the rate constant ratio $k_3^H/k_3^D$, obtained from eq 6 for the two solvents, $H_2O$ and $D_2O$. This leads to

$$k_3^H/k_3^D = \frac{\tau_f^D\alpha^D[(Q_0/Q)^H - 1]}{\tau_f^H\alpha^H[(Q_0/Q)^D - 1]} \quad (7)$$

At neutral pH, $\alpha^D = \alpha^H = 1$. The lifetime ratio, $\tau_f^D/\tau_f^H = 1.37$, is obtained from the data for L-trp (Figure 1) since quantum yields in these systems are proportional to lifetime.[2,3] In good agreement with this value, a lifetime ratio of 1.39 was obtained from studies of $I^-$ quenching of L-trp at pH 11 in $H_2O$ and $D_2O$.[20] From eq 7, $k_3^H/k_3^D = 2.7$. This is in reasonable agreement with the value 3.0 calculated by Eisinger and Navon for L-trp at higher temperatures,[4] and is characteristic of isotope effects for nonclassical proton transfer observed in other systems.[21] In the case of L-trp-L-tyr, the slightly larger quantum yield ratio observed in the plateau region compared to the peak region also indicates a similar mechanism. The intramolecular quenching of indole-3-acetamide by glycine and the large isotope effect observed also provide evidence for the proton transfer mechanism in these systems.

A shift of the fluorescence spectrum to shorter wavelengths is associated with the protonation of the amino and carboxyl groups of tryptophyl peptides. Shifts in nmr spectra[22] and in absorption spectra[23] caused by the same ionization changes for glycyl and tryptophyl peptides have been explained by inductive effects. In agreement with the absorption study, smaller shifts were observed for dipeptides when an additional peptide bond was situated between the ionizing group and the indole side chain. It is known that the fluorescence of indole in polar solvents at room temperature is shifted to longer wavelengths due to an excited state interaction with the solvent.[4,24,25] Inductive effects which perturb the electronic transitions may alter the interaction of the fluorophor with the polar solvent during the excited state and cause a shift of fluorescence spectrum.

Although the first observation that intramolecular viscosity-dependent processes are involved in the quenching of tryptophan was made by Weber,[7] Weinryb and Steiner stressed the importance of proton transfer from amino groups in determining quantum yield.[17] It has recently also been shown that the quantum yield of tryptophyl-containing compounds is markedly viscosity dependent only if the compound has a free amino group.[26]

The work reported here provides further evidence for the importance of quenching by proton transfer to a fluorophor and suggests that mechanisms of this type which may occur in proteins can be profitably studied in $D_2O$.

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# Communications to the Editor

### $\alpha$-Deuterium Isotope Effects on the Solvolyses of Norbornyl Brosylates and Synthesis of Labeled Norbornyl Derivatives[1]

*Sir:*

The nonclassical[2] and classical[3] ion theories of the behavior of norbornyl brosylates were not differentiated by earlier $\alpha$-deuterium isotope effect studies. Lee and Wong suggested two interpretations of the lower $\alpha$-isotope effect on acetolysis of *exo*-norbornyl-2-*d* brosylate (I-2-*d*) relative to *endo*-norbornyl-2-*d* brosylate (II-2-*d*).[4] In the nonclassical ion interpretation the low isotope effect could result from anchimeric assistance, which confers SN2 character to $C_2$ (III). SN2 reactions (IV) have nil $\alpha$-isotope effects.[5] Par-

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