Excited-State Proton-Transfer Reactions. A Deuterium Isotope Effect on Fluorescence

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Abstract: A large deuterium isotope effect on fluorescence emission spectra and quantum yields was observed in a number of chromophores that contain proton donor groups. In the absence of a proton donor or acceptor group, there is no appreciable isotope effect. When both the protonated (R^*H) and unprotonated (R^{*-}) excited species are fluorescent, the shape of the emission spectrum is different in H_2O and D_2O. This finding is interpreted in terms of an isotope effect on the rate of proton transfer during the excited-state lifetime in the reaction $R^*H + H_2O \rightleftharpoons R^{*-} + H_3O^+$: k_1 forward, k_2 reverse reaction rates. For 2-naphthol, k_1 is 5.29×10^7 in H_2O and 1.31×10^7 in D_2O; k_2 is 5.5×10^{10} in H_2O and 3.5×10^{10} in D_2O. These rate constants account for the larger proportion of R^{*-} emission seen in H_2O compared to D_2O upon excitation of RH. For compounds in which R^{*-} is nonfluorescent, the quantum yield is observed to be higher in D_2O than in H_2O, if a proton donor group is present on the chromophore. It is postulated that this isotope effect on quantum yield is due to a slower rate of proton transfer in D_2O to form a nonfluorescent species during the excited-state lifetime. For example, in 5-amino-1-naphthalenesulfonate, the quantum yield is 3.04-fold higher in D_2O. In contrast, the quantum yield of 1-naphthalenesulfonate is the same in H_2O and D_2O. A comparison of the fluorescence properties of a chromophore in H_2O and D_2O should prove useful in determining whether the excited state is involved in proton-transfer reactions.

Proton-transfer reactions involving molecules in electronically excited states have been studied by fluorescence spectroscopy.[1] These investigations are of interest from several points of view. First, they have shown that there are large changes in the acidities of some molecules upon electronic excitation.[2] Shifts in pK of more than 6 units have been observed in a variety of compounds. Moreover, the kinetics of some of these proton-transfer reactions have been deduced from fluorescence studies.[3] Rates of the order of 10^8 sec^{-1} have been measured. Finally, proton-transfer reactions are relevant to the interpretation of fluorescence quantum yields since they constitute a mechanism of quenching.[4]

The present study deals with a comparison of the emission properties of a number of chromophores in H_2O and D_2O. A kinetic isotope effect on fluorescence emission was expected for proton-transfer reactions involving an electronically excited species. In fact, we find a large isotope effect on the fluorescence properties of chromophores which readily participate in proton-transfer reactions. In contrast, chromophores not involved in proton-transfer reactions during the excited state typically show very similar quantum yields and emission spectra in H_2O and D_2O.

Experimental Section

Materials. Indole (MCB), 1-methylindole (K&K), L-tryptophan (Calbiochem), phenol (Mallinckrodt), anisole (Eastman), L-tyrosine (Calbiochem), and 2-naphthol (Eastman) were used without further purification. 1-Naphthalenesulfonic acid (Eastman), 5-amino-1-naphthalenesulfonic acid (Eastman), and the trisodium salts of 8-amino-1,3,6-naphthalenetrisulfonic acid (Eastman) and 1,3,6-naphthalenetrisulfonic acid (Aldrich) were recrystallized from water. The fluorescence excitation spectra of these compounds were identical with their absorption spectra, indicative of the absence of fluorescent impurities.[5] D_2O (99.88%) was purchased from Biorad. The emission properties reported here are for the chromophores dissolved in unbuffered 100% H_2O or in 98% D_2O–2% H_2O, unless otherwise indicated. Identical results were obtained in 0.001 M phosphate buffer, pH 6.8, and in unbuffered solutions.

Fluorescence Spectra. Emission spectra were obtained on a recording spectrofluorimeter to be described in detail elsewhere. The excitation source was a PEK 100-w mercury arc. The compounds were excited at a mercury line near their longest wavelength emission maxima. 2-Naphthol was excited at 321 mμ, the isosbestic point in the absorption spectra of the un-ionized and ionized species. Bausch and Lomb grating monochromators operated at a slit width corresponding to a 3.3 mμ half-band width were used for excitation and emission. The emission was detected by an RCA 1P21 photomultiplier tube for all of the compounds except phenol, tyrosine, and anisole, for which an RCA 1P28 was used. The amplified output of the photomultiplier tube was recorded on a Varian X–Y recorder which was coupled to the emission monochromator wavelength drive. The emission spectra reported here are the direct recorder tracings which have not been corrected for the variation with wavelength in the sensitivity of the detection system. Absorption spectra were taken on a Cary Model 15 recording spectrophotometer. Spectra were obtained at 20°.

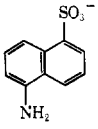
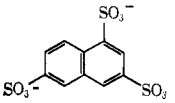
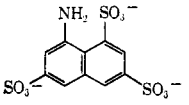
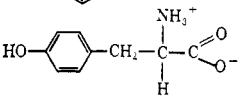
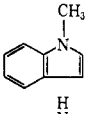
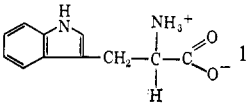
Quantum Yield Ratios. The ratio of the peak height in the emission spectrum of a compound in D_2O to that in H_2O is equivalent to the quantum yield ratio if (a) the emission spectra have the same shape when normalized and (b) the optical densities at the wavelength of excitation are identical. Quantum yield ratios were obtained in this way for all of the compounds reported here except 2-naphthol.

Results

(a) Quantum Yield. A marked deuterium isotope effect on the quantum yield of fluorescence was observed in some of the chromophores studied (Table I). The ratio of the quantum yield in D_2O to that in H_2O ranges from 1.00 to 3.70. Of the compounds studied, those that do not contain a proton acceptor or donor group at neutral pH typically show a small isotope effect. The D_2O/H_2O quantum yield ratio of 1-naphthalenesulfonate, 1,3,6-naphthalenetrisulfonate, anisole, and 1-methylindole is between 1.00 and 1.09. In contrast, a sizable deuterium isotope effect is observed when the same chromophoric systems contain

(1) For a review, see A. Weller, *Progr. Reaction Kinetics*, **1**, 189 (1961).
(2) T. Förster, *Z. Elektrochem.*, **54**, 42 (1950).
(3) A. Weller, *Z. Physik. Chem.* (Frankfurt), 3, 238 (1955).
(4) G. Weber in "Light and Life," W. D. McElroy and B. Glass, Ed., Johns Hopkins Press, Baltimore, Md., 1961, pp 82–107.
(5) G. Weber and F. W. J. Teale, *Trans. Faraday Soc.*, **54**, 640 (1958).

Table I. Deuterium Isotope Effect on the Quantum Yield of Fluorescence

Compound	Structure	Quantum yield ratio D_2O/H_2O
1-Naphthalenesulfonate		1.00
5-Amino-1-naphthalenesulfonate		3.04
1,3,6-Naphthalenetrisulfonate		1.00
8-Amino-1,3,6-naphthalenetrisulfonate		3.70
Anisole		1.05
Phenol		1.27
L-Tyrosine		1.25
1-Methylindole		1.09
Indole		1.29
L-Tryptophan		1.65

a proton donor group. Naphthalenesulfonate and naphthalenetrisulfonate exhibit particularly large isotope effects upon insertion of an amino group at a ring position. The D_2O/H_2O quantum yield ratio is then greater than 3. A smaller but significant effect of this kind is evident on comparing anisole with phenol and L-tryrosine, and also on comparing 1-methylindole with indole and L-tryptophan.

The relative quantum yield of fluorescence of 8-amino-1,3,6-naphthalenetrisulfonate in D_2O–H_2O mixtures was measured (Figure 1). The relationship between quantum yield and mole per cent D_2O is not linear for this compound. Rather, for a given increment in D_2O, there is a relatively greater increase in quantum yield at a higher mole proportion of D_2O.

(b) Emission Spectra. The emission spectra of the chromophores listed in Table I are nearly identical in H_2O and D_2O, when normalized to equal quantum yield. For example, the emission maxima of 8-amino-1,3,6-naphthalenetrisulfonate in H_2O and D_2O differ by less than 1 mμ (Figure 2).

In contrast, the emission spectrum of 2-naphthol is different in H_2O and D_2O (Figure 3), over a range

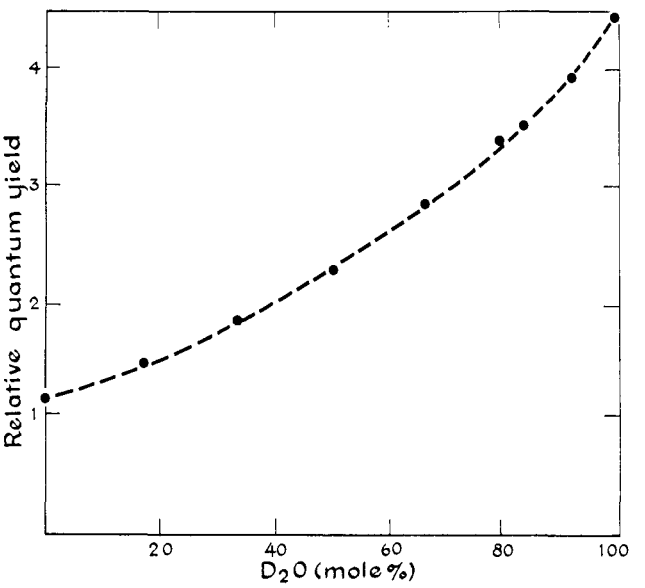


Figure 1. Relative quantum yields of fluorescence of 8-amino-1,3,6-naphthalenetrisulfonate in mixtures of H_2O and D_2O.

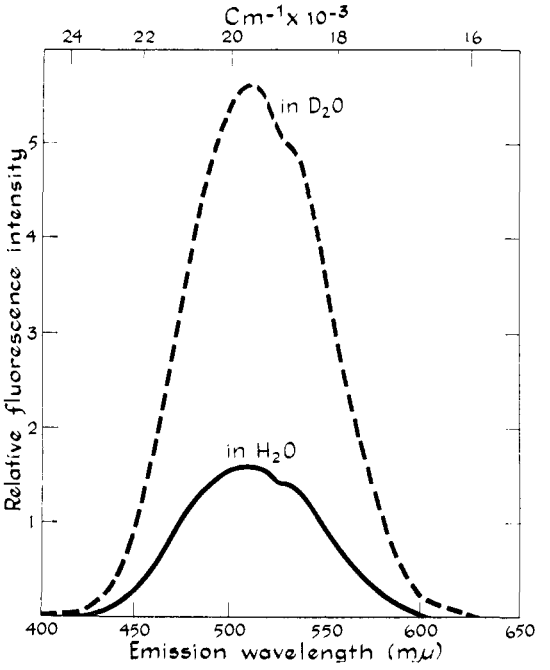


Figure 2. Fluorescence emission spectra of 8-amino-1,3,6-naphthalenetrisulfonate in H_2O (———) and D_2O (---------). The shape of the emission spectrum is the same in the two solvents, while the quantum yield is 3.7-fold higher in D_2O.

of acid pH. The emission spectrum of 2-naphthol, between pH 3 and 6, shows peaks at 352 and 416 mμ. In D_2O, the 416-mμ peak is absent, while the one at 352 mμ has a greater intensity than in H_2O. The 352-mμ peak is due to emission by un-ionized 2-naphthol, while the 416-mμ peak represents the fluorescence of the 2-naphtholate anion (Figure 4).

The differences between the emission spectra of 2-naphthol in H_2O and D_2O (Figure 3) can therefore be attributed to different proportions of fluorescence from the un-ionized and anionic species. In D_2O, the proportion of emission from the un-ionized form of 2-naphthol is greater. This effect was observed

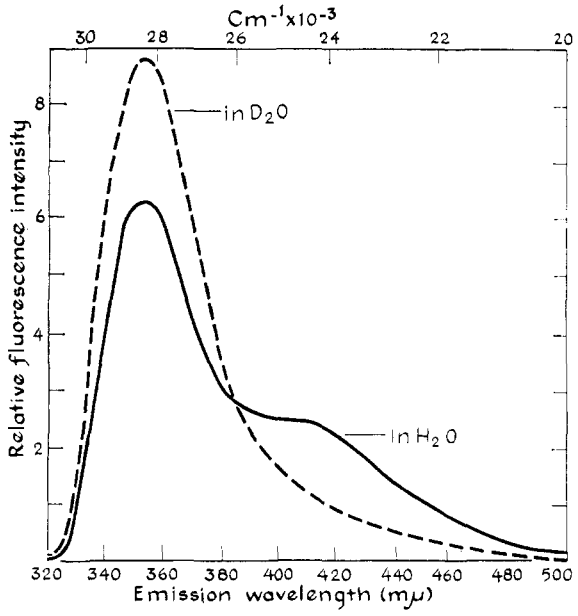


Figure 3. Fluorescence emission spectra of 2-naphthol in H_2O (———) and D_2O (--------) at pH 5. In D_2O, a greater proportion of the emission comes from un-ionized 2-naphthol and less from the 2-naphtholate ion. The differences between the spectra reflect a slower rate of proton transfer from 2-naphthol to the solvent in D_2O.

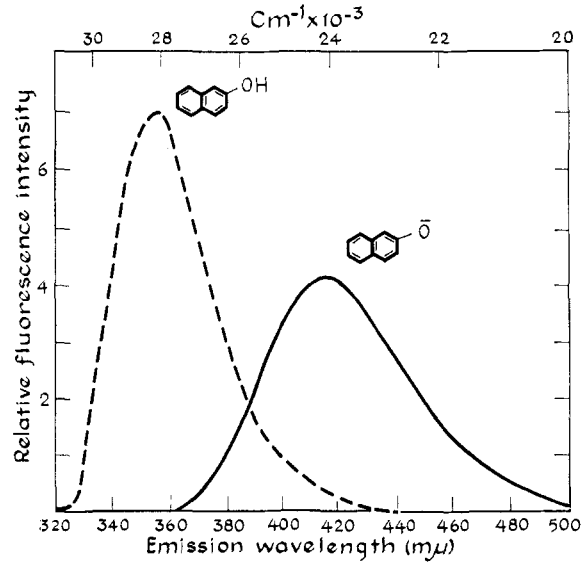


Figure 4. Fluorescence emission spectra of 2-naphthol in 1 *N* HCl (--------) and 1 *N* NaOH (———). In 1 *N* HCl the fluorescence (352-mμ peak) comes from un-ionized 2-naphthol, while in 1 *N* NaOH the fluorescence (416-mμ peak) is due to the 2-naphtholate ion. In 1 *N* HCl and in 1 *N* NaOH, the quantum yields of fluorescence and the shape of the emission spectra are nearly the same in D_2O and H_2O.

over a wide range of acid pH (Figure 5). Since the absorption spectra of 2-naphthol in H_2O and D_2O are very similar, it is evident that the isotope effect involves the excited state rather than the ground state of the chromophores.

This large isotope effect was not observed in 1 *N* HCl or in 1 *N* NaOH, where virtually all of the emission arises from a single excited-state species. In 1 *N* HCl, both H_2O and D_2O solutions of 2-naphthol show a single emission peak at 352 mμ; in 1 *N* NaOH,

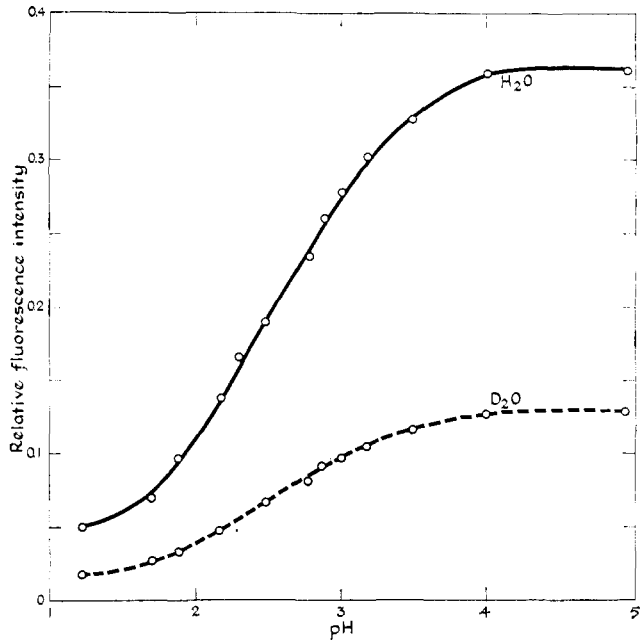


Figure 5. Relative quantum yields of 2-naphtholate anion fluorescence upon excitation of the un-ionized species as a function of pH in H_2O (———) and D_2O (--------). The ordinate is the ratio of the quantum yield obtained on excitation of RH to that obtained on direct excitation of R^- (in 1 *N* NaOH). pH values were calculated on the basis of $[H^+]$ added as HCl.

only the peak at 416 mμ is observed (Figure 4). The quantum yields are the same in H_2O and D_2O under these conditions.

Discussion

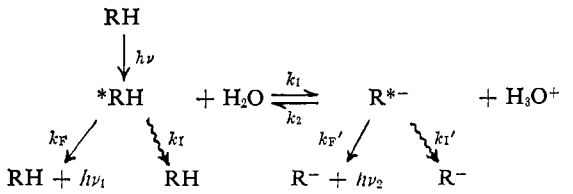
The large effect of D_2O on the fluorescence of some of the chromophores studied can be readily interpreted in terms of an *isotope effect on the rate of proton transfer during the excited-state lifetime*. The compounds investigated are of three types.

I. The chromophore does not contain a proton-donor group. For this type of compound, a sizable isotope effect is absent. 1-Naphthalenesulfonate, 1,3,6-naphthalenetrisulfonate, anisole, and 1-methylindole are examples of this type of chromophore.

II. The chromophore contains a proton-donor group. Only the un-ionized species is fluorescent. Here, the isotope effect is expressed in terms of a *change in quantum yield*. Emission from the un-ionized species competes with a quenching reaction whose rate is faster in H_2O than in D_2O. 5-Amino-1-naphthalenesulfonate, 8-amino-1,3,6-naphthalenetrisulfonate, phenol, L-tyrosine, indole, and L-tryptophan illustrate this category.

III. The chromophore contains a proton-donor group. In addition, both the un-ionized and ionized forms of the chromophore are fluorescent. For these compounds, the isotope effect is expressed in terms of a *change in the shape of the emission spectrum*, since the rate of proton transfer during the excited state determines the proportion of emission from the un-ionized and ionized species. 2-Naphthol is an example of this type of chromophore.[1,3]

Some quantitative features of the isotope effect can be deduced from the following scheme.



RH is the un-ionized form of the chromophore in the ground state; R*H, in the first excited singlet state. For simplicity, it is assumed that the concentration of the ionized form in the ground state is negligible. Experimentally, this was achieved by taking fluorescence measurements at a pH far from the ground-state pK. The rate constants for fluorescence of R*H and R*$^-$ are k_F and k_F', respectively. The corresponding rate constants for nonradiative transitions to the ground state are k_I and k_I'. The pseudo-first-order rate constant for proton transfer to H_2O is k_1, while the second-order rate constant for protonation of R*$^-$ by H_3O^+ is k_2.

When R*H and R*$^-$ are both fluorescent, the rate constants k_1 and k_2 can be directly determined. Weller[6] has shown that the quantum yield q of R*$^-$ emission observed on excitation of RH and subsequent proton transfer to form R*$^-$ is

$$q = Q' \frac{k_1\tau}{1 + k_1\tau + k_2[H_3O^+]\tau'} \qquad (I)$$

where Q' is the quantum yield of R*$^-$ emission upon direct excitation of R$^-$, $\tau = (k_F + k_I)^{-1}$, and $\tau' = (k_F' + k_I')^{-1}$. To compare k_1 in H_2O and D_2O, it is simplest to consider the pH range in which the back reaction of R*$^-$ to R*H does not occur, *i.e.*, when $k_2[H_3O^+]\tau' << (1 + k_1\tau)$. This condition is met at pH 5 for 2-naphthol in both H_2O and D_2O, as shown in Figure 5. The quantum yield of R*$^-$ emission is then

$$q = Q' \frac{k_1\tau}{1 + k_1\tau} \qquad (II)$$

and the forward rate constant for proton transfer is

$$k_1 = \frac{(q/Q')}{1 - (q/Q')} \frac{1}{\tau} \qquad (III)$$

τ is 11 nsec,[6] and (q/Q') is 0.368 in H_2O and 0.126 in D_2O at 20° (Figure 5). Thus the forward rate constant is 5.29×10^7 in H_2O and 1.31×10^7 in D_2O.

The rate constant k_2 for the back-reaction can be obtained from the dependence of R* emission on pH between 2 5 and 5.0. Rearrangement of eq I gives

$$\frac{Q'}{q} = 1 + \frac{1}{k_1\tau} + \frac{k_2\tau'}{k_1\tau}[H_3O^+] \qquad (IV)$$

A plot of Q'/q *vs.* $[H_3O^+]$ gives an intercept equal to $[1 + (1/k_1\tau)]$ and a slope equal to $(k_2\tau')/(k_1\tau)$. τ' is 8.1 nsec.[6] When the data in Figure 5 are plotted in this way, k_2 is found to be 5.5×10^{10} in H_2O and 3.5×10^{10} in D_2O.

Thus, the k_H/k_D ratio for the forward reaction R*OH + $H_2O \rightarrow$ R*O$^-$ + H_3O^+ is 4.04, while the k_H/k_D ratio for the back-reaction R*O$^-$ + $H_3O^+ \rightarrow$ R*OH + H_2O is 1.57. The different magnitude of the kinetic isotope effect on the two rate constants indicates that there is a change in the excited-state pK* on going from H_2O to D_2O. The pK* values determined from the ratio k_1/k_2 are 3.0 in H_2O and 3.4 in D_2O. The lower pK* observed in H_2O agrees with the generalization that protolytic dissociation constants of weak acids are almost always larger in H_2O than in D_2O.[7] The present experimental results confirm the pK* values calculated by Wehry and Rogers[7] from absorption spectra of 2-naphthol in H_2O and D_2O. They calculated a pK* of 3.0 in H_2O and 3.5 in D_2O.

For type II compounds, the analysis is less definitive. The quantum yield of R*H emission when R* is nonfluorescent ($k_I' >> k_F'$) and is quenched before the back-reaction to R*H can occur ($k_I' >> k_2[H_3O^+]$) is

$$Q = \frac{k_F}{k_F + k_1 + k_I} \qquad (V)$$

Thus, the formation of nonfluorescent R*$^-$ by proton transfer during the excited state is an important mechanism of quenching only if k_1 is comparable in magnitude to $(k_F + k_I)$. For type II compounds, k_1 has not been directly measured, but its magnitude has been inferred from the difference in wavelength between the maxima in the absorption spectra of the un-ionized and ionized forms of the molecule. For example, Feitelson[8] has estimated that the excited-state pK* of phenol is about 5 and that the forward rate constant k_1 is likely to be of the order of 10^5. Since $(k_F + k_I)$ for phenol is of the order of 10^8, it is evident that formation of the nonfluorescent phenolate ion by proton transfer during the excited state lifetime is too slow to be important in the quenching of the fluorescence of phenol.[8] However, it is likely that complete proton transfer to form an ionized R*$^-$ is not the only mechanism by which quenching occurs. Partial proton transfer to form a nonfluorescent species intermediate between R*H and R*$^-$ may suffice. The observed isotope effect on quantum yield (Table I) could then be due to a slower rate formation in D_2O of this postulated nonfluorescent intermediate.

Let k_H be the rate constant of this assumed partial proton-transfer reaction in H_2O and k_D be the rate constant in D_2O. Then, the ratio of the quantum yield in D_2O, Q_D, to that in H_2O, Q_H is given by

$$\frac{Q_D}{Q_H} = \frac{k_F + k_I + k_H}{k_F + k_I + k_D}$$

if it is assumed that the intrinsic rate constant of fluorescence k_F and the sum of the rate constants of all other processes of internal conversion k_I are independent of isotope. This assumption is supported by the fact that Q_D/Q_H is between 1.00 and 1.09 for those chromophores studied which do not contain a readily dissociable proton. In addition, nanosecond pulse experiments carried out in this laboratory have shown that the excited-state lifetimes in D_2O and H_2O are proportional to quantum yields, indicating that k_F is independent of isotope in these compounds. Thus, the ratio Q_D/Q_H is expected to vary between 1 and k_H/k_D. If either the quantum yield is high or if internal conversion is faster than proton transfer as a mechanism of quenching, Q_D/Q_H will tend toward

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(7) E. L. Wehry and L. B. Rogers, *J. Am. Chem. Soc.*, **88**, 351 (1966).

(8) J. Feitelson, *J. Phys. Chem.*, **68**, 391 (1964).

1. On the other hand, Q_D/Q_H will approach k_H/k_D if both the quantum yield is low and proton transfer is the predominant quenching mechanism. In this study, the quantum yields[9,10] are sufficiently low so that k_F is less than $(k_I + k_H)$. The quantum yield[9,10] of fluorescence of 8-amino-1,3,6-naphthalenetrisulfonate is 0.15; phenol, 0.22; tyrosine, 0.21; indole, 0.45; and tryptophan, 0.20. The different Q_D/Q_H ratios observed for these chromophores therefore reflect differences either in their k_H/k_D ratios or in the relative magnitudes of k_H and k_I.

These results suggest that a comparison of the *fluorescence properties of a chromophore in H_2O and D_2O is useful in determining whether the excited species is involved in proton-transfer reactions.* This criterion is valid to the extent that deuteration of an aromatic chromophore or of its solvent has little effect on fluorescence in the absence of proton-transfer reactions. This assumption is supported by the findings of Lim and Laposa[11] that the quantum yield of fluorescence of several aromatic hydrocarbons is the same for the perdeuterated and perprotonated compounds. Also, there is no isotope effect on the fluorescence lifetimes of these compounds.[12] Isotope effects on fluorescence have been described for only two kinds of chromophores apart from the proton-transfer category discussed here. Kropp and Windsor[13] found that fluorescence quantum yields and lifetimes of rare-earth complexes in solution are markedly enhanced in deuterated solvents. Johnson, Logan, and Ross[14] observed that the fluorescence intensity of perdeuterated azulene is greater than that of perprotonated azulene. It should be noted that the fluorescence of azulene is in itself anomalous since azulene emits from the second excited singlet state. Large isotope effects on phosphorescence have been found following the initial observations of Hutchison and Mangum[15] and of Wright, Frosch, and Robinson.[16] Thus, it appears that only azulene and rare-earth ions are exceptions to the generalization that a large isotope effect on fluorescence is only observed if the excited chromophore participates in proton-transfer reactions.

The quantum yield of tyrosine and tryptophan emission in proteins is almost always lower than that of the free amino acids.[17,18] The probable involvement of proton transfer as a mechanism of quenching has been proposed by Weber.[4,18] The carboxylate groups of glutamic and aspartic acid residues are likely proton acceptors in proteins. Weber and Rosenheck[18] have shown that the quantum yield of tyrosine emission in copolymers of glutamic acid and tyrosine increases threefold on O-methylation of tyrosine. The present findings of an isotope effect on the fluorescence of tyrosine and tryptophan reinforces their conclusions concerning the importance of proton-transfer reactions in determining the quantum yield. Furthermore, preliminary experiments in this laboratory reveal an isotope effect on the fluorescence of these chromophores in proteins.

The present work also affords an interpretation of the marked dependence on solvent of the quantum yield of 1-anilino-8-naphthalenesulfonate (ANS).[19,20] ANS has a quantum yield of 0.004 in H_2O, 0.37 in ethanol, and 0.63 in 1-octanol. When bound to a nonpolar site in a protein such as apomyoglobin,[20] its quantum yield increases to 0.98. Recently, a D_2O effect on ANS fluorescence has been reported.[21] These observations can be interpreted in terms of a proton-transfer reaction involving the excited state of ANS.

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