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Fluorescent Derivatives of Nitrotyrosine. Model Compounds for Fluorescent Reporter Groups in Proteins*

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ABSTRACT: The synthesis and spectral properties of the fluorescent derivatives of ethyl α-*N*-acetyl-3-aminotyrosinate and α-*N*-acetyl-3-aminotyrosinamide resulting from conjugation with 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) are reported. The compounds, ethyl α-*N*-acetyl-3-(1′-dimethylaminonaphthalene-5′-sulfonamido)tyrosinate (EADT) and α-*N*-acetyl-3-(1′-dimethylaminonaphthalene-5′-sulfonamido)tyrosinamide (ADTA), were synthesized starting with 3-nitro-L-tyrosine. After appropriate substitution on the α-amino and carboxyl groups, the nitro group was reduced to the corresponding aromatic amine, which was then specifically reacted with dansyl chloride at pH 5.0. Both EADT and ADTA behave spectrally as would be expected for a dansylated amine. Both have a molar absorptivity in 60% (v/v) ethanol of *ca.* 4600 M^{-1} cm^{-1} in the 320- to 340-nm region, and a half-titration of the dimethylamino groups occurs at approximately pH 3.0. The fluorescence excitation spectra coincide with the absorption spectra at wavelengths above 320 nm, but the excitation extinction is considerably lower at shorter wavelengths. Both the fluorescence excitation and emission spectra are sensitive to solvent polarity; the excitation maxima shift to shorter wavelengths and the emission maxima to longer wavelengths (by as much as 30 nm) in going from ethanol to water solvent. Accompanying the increase in wavelength of fluorescence emission, the fluorescence quantum efficiency decreases by about 80% for both EADT and ADTA.

The use of EADT and ADTA as model compounds of fluorescent dansyltyrosyl "reporter" groups in proteins is proposed.

The exposure and reactivity of tyrosyl residues in proteins have been probed by several physical and chemical techniques. Among the most widely used methods have been spectrophotometric (Leach and Scheraga, 1960) and fluorometric measurements (Teale, 1960; Cowgill, 1965a,b, 1966, 1968; Edelhoch *et al.*, 1968), difference spectra (Laskowski, 1966), iodination (Dube *et al.*, 1964a,b, 1966), acetylation (Simpson *et al.*, 1963; Riordan *et al.*, 1965), and reaction with cyanuric fluoride (Kurihara *et al.*, 1963; Gorbunoff, 1967, 1969) and with diazonium-1*H*-tetrazole (Sokolovsky and Vallee, 1966, 1967). Riordan and coworkers (Riordan *et al.*, 1966, 1967; Riordan and Christen, 1968) introduced tetranitromethane (TNM[1]) as a reagent that preferentially nitrates tyrosyl and oxidizes cysteinyl residues in several proteins. Its usefulness in probing the functional role of tyrosyl residues in certain enzymes has been demonstrated (Sokolovsky *et al.*, 1966; Riordan *et al.*, 1967; Johansen *et al.*, 1967; Cuatrecasas *et al.*, 1968; Kenner *et al.*, 1968; Holeysovsky *et al.*, 1969)

Nitration of tyrosyl residues has the advantage over most methods in that further highly specific chemical derivatives of the nitrotyrosyl residues can be made after reduction of the nitro to the amino group, the only major limitation being the specificity of the initial nitration reaction. 3-Nitrotyrosyl residues are mildly and specifically reduced with $Na_2S_2O_4$ to 3-aminotyrosyl residues. The p*K* of 3-aminotyrosine in proteins is below pH 5.0 (Sokolovsky *et al.*, 1967). This low hydrogen ion dissociation range allows for specific modification of the aromatic amino groups with numerous reagents, *e.g.*, selective acetylation, diazotization, or dansylation. Following limited nitration with TNM and reduction

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[1] The following abbreviations are used: TNM, tetranitromethane; dansyl chloride, 1-dimethylaminonaphthalene-5-sulfonyl chloride; EADT, ethyl α-*N*-acetyl-3-(1′-dimethylaminonaphthalene-5′-sulfonamido)tyrosinate; ADTA, α-*N*-acetyl-3-(1′-dimethylaminonaphthalene-5′-sulfonamido)tyrosinamide.

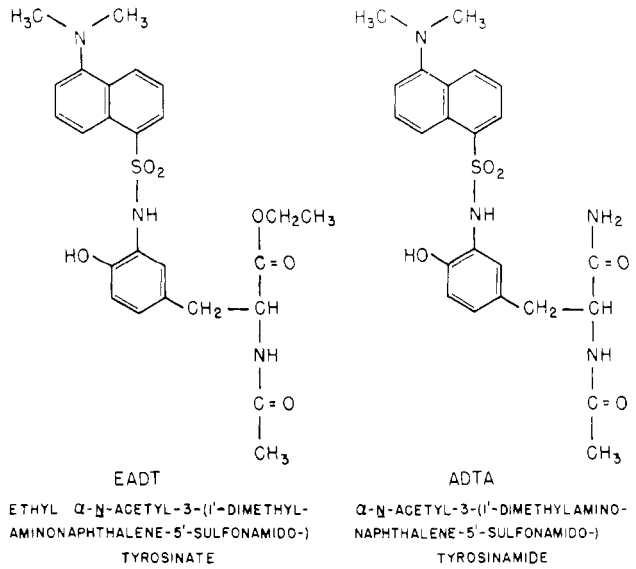

FIGURE 1: Structure of EADT and ADTA.

with $Na_2S_2O_4$, bovine trypsin and trypsinogen were reacted with 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) to produce proteins specifically labeled with a fluorophore. The results of these investigations are presented in the accompanying paper (Kenner and Neurath, 1971).

To facilitate interpretation of data obtained with proteins labeled with fluorescent dansyltyrosyl "reporter" groups (Burr and Koshland, 1964), two model compounds were synthesized and several of their spectral characteristics are described in this communication. Since the fluorescence of tyrosine is largely dependent on its ionization state and the presence or absence of carboxyl modification (Edelhoch *et al.*, 1968), two dansyltyrosine model compounds representing two carboxyl modifications were synthesized and characterized: the ethyl ester, ethyl α-*N*-acetyl-3-(1'-dimethylaminonaphthalene-5'-sulfonamido)tyrosinate (EADT), and the corresponding amide, α-*N*-acetyl-3-(1'-dimethylaminonaphthalene-5'-sulfonamido)tyrosinamide (ADTA). In general, EADT and ADTA have features desired of a "reporter" group placed to signal information about the microenvironment of tyrosyl side chains in proteins, *i.e.*, their dansyl fluorescence shows a high quantum efficiency which is extremely sensitive to solvent polarity.

Experimental Section

Synthesis of EADT and ADTA. 3-Nitro-L-tyrosine (K & K Laboratories) was converted into the ethyl ester in ethanol saturated with dry HCl. The hydrochloride of this ester (0.0125 mole) was α-*N*-acetylated with acetic anhydride (0.0125 mole) in 200 ml of dry pyridine containing 2 equiv of triethylamine. Acetylation took place at 0° for 2 hr and then at room temperature overnight. Ethyl α-*N*-acetyl-3-nitro-L-tyrosinate was crystallized twice from ethanol–water (yield 2.7 g). This derivative (0.0086 mole) was reduced with $Na_2S_2O_4$ (0.0430 mole) to the corresponding aromatic amine in 100 ml of 0.05 M pyridine–acetate, pH 5.0, at room temperature for 1 hr. The resulting compound was taken to dryness and extracted into absolute ethanol. The aminotyrosine derivative was crystallized twice from ethanol–water as the hydrochloride (yield 1.5 g). Ethyl α-*N*-acetyl-3-aminotyrosinate hydrochloride (0.005 mole) was dansylated with 0.005 mole of freshly prepared dansyl chloride (Horton and Koshland, 1965) in 30 ml of acetone and 200 ml of 0.05 M pyridine–acetate, pH 5.0, protected from light. After stirring at room temperature for 18 hr, the product, ethyl α-*N*-acetyl-3-(1'-dimethylaminonaphthalene-5'-sulfonamido)tyrosinate (EADT) (Figure 1), layered on the walls of the reaction vessel as a viscous green oil. The oil was extracted four times with 50-ml portions of 5% $KHCO_3$. The compound was crystallized twice (with considerable difficulty) as the free base from ethyl acetate–water–diethyl ether (yield 1.0 g, mp 88–90°). EADT can easily be crystallized from ethanol–ether as the hydrochloride.

α-*N*-Acetyl-3-(1'-dimethylaminonaphthalene-5'-sulfonamido)tyrosinamide (ADTA) (Figure 1) was prepared by converting ethyl α-*N*-acetyl-3-nitro-L-tyrosinate into the corresponding amide by ammonia in ethanol. Reduction of the nitro group and reaction with dansyl chloride were carried out under conditions identical with those used for the synthesis of EADT. ADTA was easily crystallized from ethanol–ether as the monohydrochloride. Yield from 217 g of α-*N*-acetyl-3-amino-L-tyrosinamide starting material was 0.9 g of ADTA (mp 118–121°).

ADTA and EADT both gave single fluorescent spots on thin-layer chromatography (chloroform–ethanol–formic acid, 70:30:2). Elemental analyses gave the expected composition for both compounds (Table I).

TABLE I: Elemental Analyses of EADT and ADTA.[a]

Element	EADT (Mol Wt 499.3) Expected (%)	EADT (Mol Wt 499.3) Found (%)	ADTA (Mol Wt 506.6) Expected (%)	ADTA (Mol Wt 506.6) Found (%)
C	60.08	60.06	54.48	53.11
H	5.85	5.92	5.33	5.66
O[b]	19.22	19.22	15.79	15.87
N	8.41	8.27	11.06	11.32
S	6.42	6.53	6.33	6.47
Cl			7.01	7.55

[a] Analyses were performed by A. Bernhardt, 5251 Elbach ueber Engelskirchen, West Germany. [b] By difference.

Spectral Measurements. A Turner spectrophotometer–spectrofluorometer Spectro 210 was utilized for all absorption, excitation, and emission spectra. Molar absorptivities determined on the Turner 210 and on a Cary 16 spectrophotometer agreed within 2%. Quantum efficiencies were evaluated by the use of the relationship

$$Q = Q_s \frac{\int A_u d\lambda \; \lambda_{exs} \epsilon_s C_s}{\int A_s d\lambda \; \lambda_{exu} \epsilon_u C_u}$$

(Turner, 1964), where $\int A d\lambda$ is the area under the emission curve as measured by planimetry. The subscripts u and s refer to the unknown and standard, respectively. Quinine bisulfate (Matheson Coleman and Bell), recrystallized several times from water, was used as the fluorescent standard to calibrate the instrument. The quantum yield of quinine bisulfate was taken as 0.57 at an excitation wavelength of 348 nm (Turner, 1966a). For determination of excitation

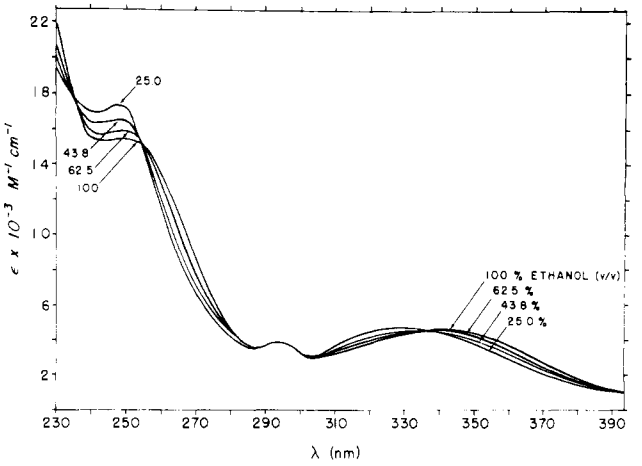

FIGURE 2: Absorption spectra of EADT in ethanol–water mixtures. The percentages of ethanol in water (v/v) are indicated on the curves.

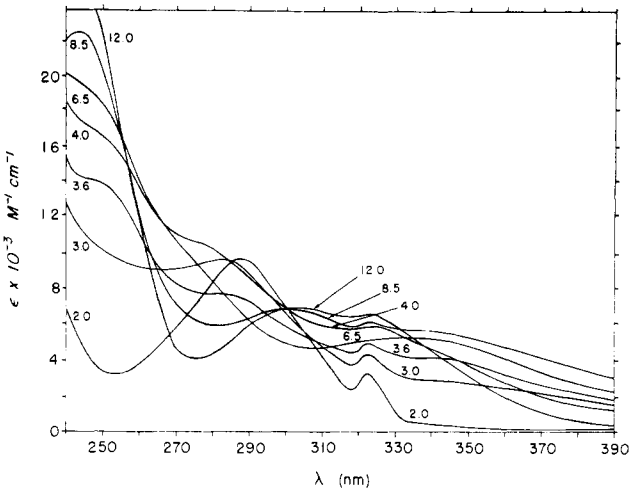

FIGURE 4: Absorption changes in EADT with changes in pH. The pH values are indicated on the curves. Each solution contained 20% (v/v) ethanol.

spectra and quantum yields, $A_{\lambda_{exs,u}}{}^{1cm}$ was usually less than 0.01. Correction of absorption effects in recording excitation spectra were made according to Turner (1966b). The band width selected on the excitation monochromator for determining excitation and absorption spectra was 2.5 nm. Emission spectra were measured with band widths on the excitation and emission monochromators of 10 nm or less.

A potassium acetate buffer (0.05 M) was used for most fluorescent measurements below pH 6.5 and Tris (Trizma base, Sigma Chemical Co.), 0.05 M, for buffers higher than pH 6.5. HCl and NaOH were used for extremes of pH. All fluorescence measurements were made at 25°.

Results

Absorption Spectra. The absorption properties of EADT in ethanol–water mixtures are shown in Figure 2. Aminotyrosine has an absorption maximum at 288 nm (ϵ 2800 M^{-1} cm^{-1}) in the pH region 7.4–9.0 (Sokolovsky *et al.*, 1967). Thus the absorption band at 294 nm (ϵ 3800 M^{-1} cm^{-1}) probably arises almost entirely from absorption by the aminotyrosine component. Both the tyrosine and 1-dimethylaminonaphthalene-5-sulfonamido groups (Lagunoff and Ottolenghi, 1966) contribute to extinction below 280 nm with an isosbestic point at 254 nm (ϵ 15,000 M^{-1} cm^{-1}). Wavelength of maximum absorption of the 1-dimethylaminonaphthalene-5-sulfonamido group in the 320- to 360-nm region depends on solvent polarity, varying between 328 nm for 25% (v/v) ethanol and 340 nm for 100% ethanol. The molar absorptivity in the 320- to 360-nm region is essentially constant for all concentrations of ethanol (ϵ 4600 M^{-1} cm^{-1}).

Absorption spectra of ADTA in ethanol–water mixtures are illustrated in Figure 3. The spectra are similar to the corresponding spectra for EADT, but there are some significant differences. Absorption in the aminotyrosine band is high (ϵ 4700 M^{-1} cm^{-1}) compared with EADT, and the absorption maximum of ADTA is 286 nm compared with 292 nm for EADT. ADTA has an isosbestic point at 256 nm (ϵ 13,100 M^{-1} cm^{-1}). The molar absorptivity of ADTA in the 320- to 360-nm region is 4500 M^{-1} cm^{-1} in 100% ethanol and 4200 M^{-1} cm^{-1} in 23% (v/v) ethanol. The wavelength of maximum absorption in this region decreases from 338 nm in 100% ethanol to 330 nm in 23% (v/v) ethanol.

Spectrophotometric pH Titrations. The titratable groups of EADT (Figure 4) show a more complex titration behavior

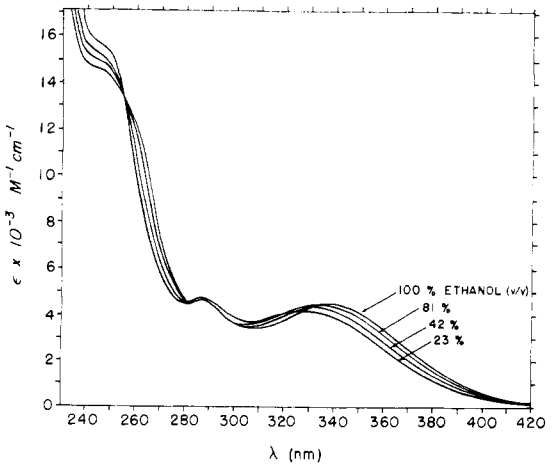

FIGURE 3: Absorption spectra of ADTA in ethanol–water mixtures. The percentages of ethanol in water (v/v) are indicated on the curves.

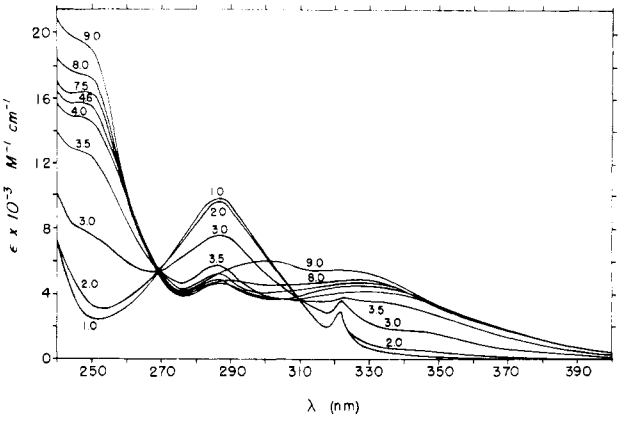

FIGURE 5: Absorption changes in ADTA with changes in pH. The pH values are indicated on the curves. Each solution contained 20% (v/v) ethanol.

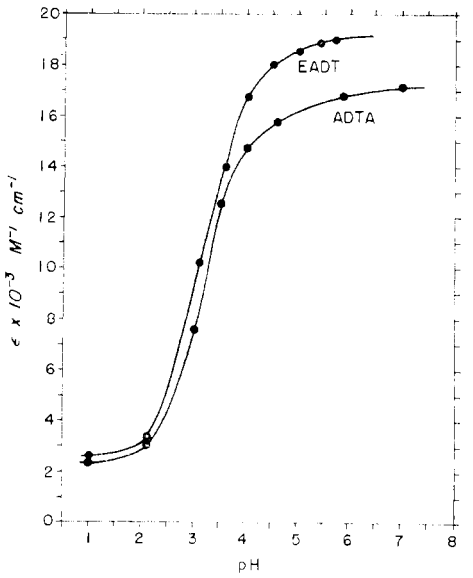

FIGURE 6: Spectrophotometric pH titration of ADTA and EADT at 250 nm. The molar absorptivity at 250 nm was measured in each case as a function of pH. Each solution contained 20% (v/v) ethanol.

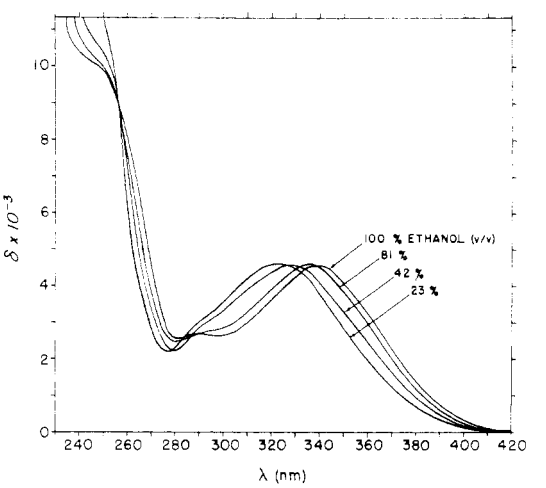

FIGURE 8: Excitation spectra of ADTA in ethanol–water mixtures. The excitation spectra were measured using a band width of 2.5 nm on the excitation monochromator and 10 nm for the emission monochromator set at 540 nm. δ is the excitation extinction normalized to ϵ = 4600 M^{-1} cm^{-1} in the 320- to 340-nm region. The percentage ethanol in water (v/v) is indicated on the curves.

than ADTA (Figure 5), especially in the region of tyrosine absorption. Both compounds have a molar absorptivity of approximately 6000 M^{-1} cm^{-1} in the 320–330 nm region for unprotonated forms, but EADT absorbs considerably more (*ca.* 20%) in the 240- to 260-nm region. The protonated forms (pH 2) have nearly identical absorption spectra. ADTA has an isosbestic point at 269 nm (ϵ 5400 M^{-1} cm^{-1}). A plot of the molar absorptivity of EADT and ADTA in 20% (v/v) ethanol at 250 nm as a function of pH (Figure 6) gives a half-titration value of approximately 3.1 for the dimethylamino group of both compounds.

Fluorometric pH Titration. Fluorometric titration of ADTA in 20% (v/v) ethanol (Figure 7) confirmed the half-titration value of approximately 3 observed spectrophotometrically and also showed that maximum quantum yields can be obtained between pH 4 and 6. Ionization of the tyrosyl hydroxyl group (p*K* 9.2) is probably responsible for the pronounced quenching of fluorescence at higher pH values (Edelhoch, 1968; White, 1959; Cowgill, 1965b).

Comparisons of Excitation and Absorption Spectra. Figure 8 shows an ethanol–water series of corrected excitation spectra for ADTA normalized in the 320- to 340-nm region to ϵ 4600 M^{-1} cm^{-1}. The excitation maxima shift to a shorter wavelength in going from 100% ethanol (λ_{max} 340 nm) to 23% ethanol (λ_{max} 323 nm). An excitation isosbestic point is observed at 256 nm. Both the isosbestic point and wavelength shifts are almost identical with those observed for absorption spectra (Figure 3). Excitation spectra of EADT depend on solvent composition similar to that of ADTA. Figure 9 compares the corrected excitation and absorption

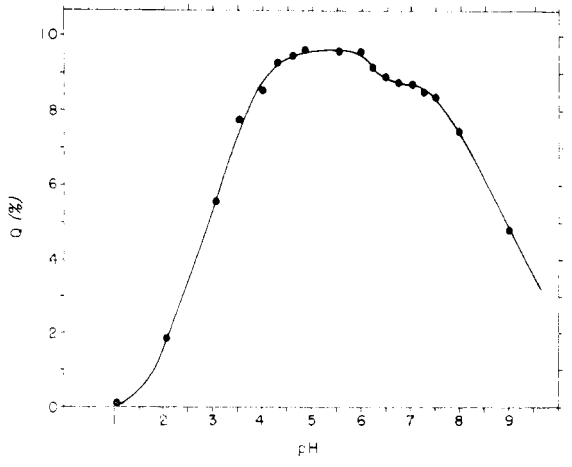

FIGURE 7: Fluorometric pH titration of ADTA. The quantum efficiencies, *Q* (%), were measured using exciting light at 335 nm. A band width of 10 nm was used for both the emission and excitation monochromators. Each solution contained 23% (v/v) ethanol.

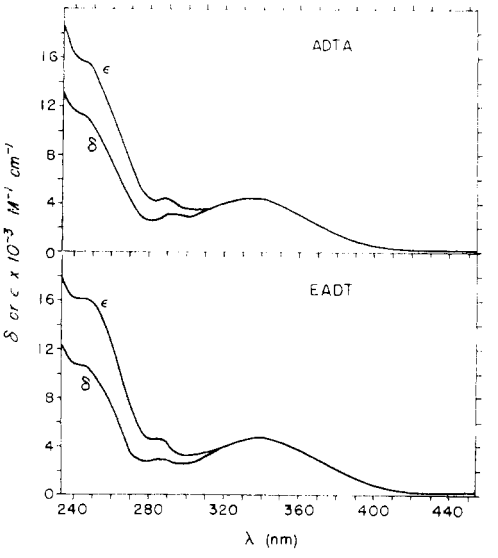

FIGURE 9: Comparison of excitation and absorption spectra of ADTA and EADT. The absorption and excitation spectra were measured in 60% (v/v) ethanol. The corrected excitation extinction, δ, is normalized to the molar absorptivity, ϵ, at 340 nm. The excitation spectra were measured using a band width of 2.5 nm on the excitation monochromator and 10 nm on the emission monochromator set at 540 nm for ADTA and 550 nm for EADT.

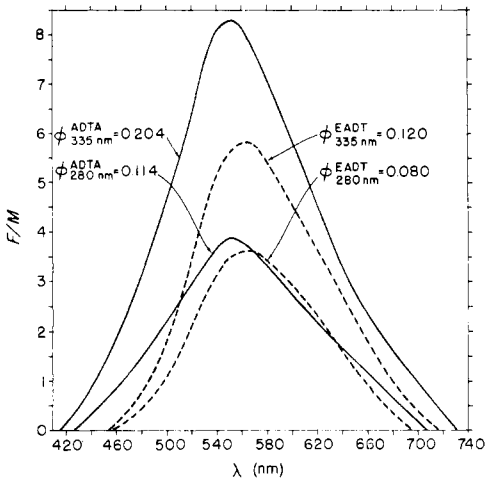

FIGURE 10: Corrected fluorescence emission spectra of EADT and ADTA. F/M is the fluorescence intensity corrected per unit band width divided by the fluorophore concentration. The excitation light was either 335 or 280 nm. The quantum efficiencies, Q, with each excitation light are indicated. The measurements were made in 60% (v/v) ethanol. The emission spectra were recorded using a band width of 10 nm on both the excitation and emission monochromators.

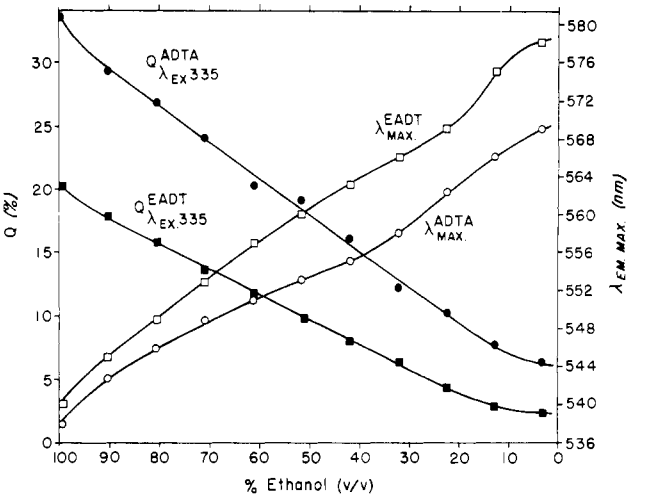

FIGURE 11: Change in fluorescence parameters with change in solvent composition. The quantum efficiency, Q (%), and wavelength of maximum fluorescence emission, $\lambda_{em\ max}$ (nm), were determined for EADT and ADTA in ethanol–water mixtures. Excitation in each case was at 335 nm. The measurements were made using band widths of 10 nm on both the excitation and emission monochromators.

spectra of ADTA and EADT in 60% (v/v) ethanol. ϵ is the molar absorptivity and δ the corrected excitation spectrum normalized to ϵ at 335 nm.

Fluorescence Emission Spectra. Fluorescence emission spectra of EADT and ADTA in 60% (v/v) ethanol are plotted in Figure 10. F/M is the fluorescence intensity corrected per unit band width divided by the molarity of the fluorophore. For direct comparison, the two sets of curves are plotted on the same scale. The exciting light was either 335 or 280 nm. The following quantum yields were calculated: $Q_{\lambda_{ex335}}{}^{ADTA} = 0.204$; $Q_{\lambda_{ex280}}{}^{ADTA} = 0.114$; $Q_{\lambda_{ex335}}{}^{EADT} = 0.120$; $Q_{\lambda_{ex280}}{}^{EADT} = 0.080$. As suggested by the identity of the absorption and excitation spectra at wavelengths greater than 320 nm (Figure 9), quantum efficiencies are constant for both compounds using exciting light longer than 320 nm.

Effect of Solvent on Quantum Efficiency. The effect of solvent composition on quantum efficiencies of EADT and ADTA is shown in Figure 11. The quantum yield of EADT is considerably lower than of ADTA in all solvents. Figure 11 illustrates that both model compounds are sensitive to solvent polarity: the wavelength of maximum emission for EDTA is 540 and for ADTA 538 nm. With increasing polarity there is a decrease in quantum efficiency and a concomitant increase in the fluorescence emission maximum.

Discussion

The molar absorptivities of EADT and ADTA (ϵ 4600 M^{-1} cm^{-1} in 60% ethanol) in the 300- to 400-nm band (Figures 2 and 3) are close to those reported by Weber (1952) for several dansyl derivatives, *viz.*, the sulfonamide (4020 M^{-1} cm^{-1}), the *N*-phenylsulfonamide (4400 M^{-1} cm^{-1}), and the *N*-benzylsulfonamide (4460 M^{-1} cm^{-1}). Whereas solvent polarity has little effect on the magnitude of the molar absorptivity of EADT and ADTA, absorption and excitation spectra depend significantly on solvent polarity (Figures 2, 3, and 8).

Excitation and absorption spectra of most fluorescent compounds are identical (Weber and Teale, 1959), but differences between these two types of spectra are expected for a complex substance containing two chromophores with different absorption and emission characteristics. The differences between the corrected excitation and the absorption spectra for EADT and ADTA (Figure 9) in the region below 310 nm are due to tyrosine absorption. Thus the differences for the quantum efficiencies at either 280- or 335-nm excitation light for each compound can be rationalized as a low efficiency energy transfer from tyrosine to the dansyl group.

Spectrophotometric and fluorometric pH titrations of both EADT and ADTA reveal a half-titration of the dimethylamino group at approximately pH 3 (Figures 6 and 7). This is about 1.5 pH units below the pK of 1-dimethylaminonaphthalene-5-sulfonic acid and 1 pH unit below that of dansyllysine (Lagunoff and Ottolenghi, 1966). The origin of the weak, unexpected shoulder on the fluorometric pH titration curve between pH 6.5 and 7.5 (Figure 7) is not clear, but it evidently arises from weak charge interactions resulting from the particular solution conformation of the compound. A similar pH dependence of fluorescence was also observed for dansyltyrosyl trypsinogen (Kenner and Neurath, 1971).

To be of use as a fluorescent reporter group in proteins, the label must be highly sensitive to some indicator of local environment, *e.g.*, polarity. Measurements of the effects of changing solvent composition, *i.e.*, solvent polarity, suggest that dansyltyrosyl residues should be useful as fluorescent reporter groups. The quantum efficiency decreases by more than 80% and the wavelength of fluorescence emission increases by approximately 30 nm for both EADT and ADTA in going from ethanol to water (Figure 11). EADT is more sensitive than ADTA to the dipolar character of the solvent, but the quantum efficiency of EADT is only about one-half that of ADTA. In general, the ethyl ester derivatives of tyrosine compounds are more sensitive to localized deactivating processes than amide derivatives (Edelhoch *et al.*, 1968), but the percentage and direction of fluorescence change in EADT and ADTA with change in solvent composition are very nearly the same. Thus these two dansyltyrosine fluorophores are suggested as model compounds for fluores-

cence studies on proteins dansylated at the aminotyrosine sites. A study of dansyltyrosyl trypsin and trypsinogen is reported in the accompanying paper (Kenner and Neurath, 1971).

Acknowledgments

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