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Synthesis and Characterization of Two Fluorescent Sulfhydryl Reagents†

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ABSTRACT: Two fluorescent sulfhydryl reagents, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (1,5-I-AEDANS) and the 1,8 isomer (1,8-I-AEDANS), were synthesized. Although sensitive to photocatalyzed degradation, these reagents readily react with thiol compounds and sulfhydryl groups in proteins yielding photostable covalent derivatives. The synthesis, fluorescence spectra, quantum yields, and lifetimes of model compounds in various solvents are presented in this paper. A following paper will report some of the general properties of the protein conjugates.

The use of fluorescent probes in protein studies is now well established. Since the introduction of fluorescein isocyanate (Creech and Jones, 1941) and dansyl chloride (Weber, 1952), a variety of covalent and noncovalent probes have been developed and applied to the elucidation of various properties of proteins (Steiner and Edelhoch, 1962; Stryer, 1968; Brand and Gohlke, 1972). With a few exceptions, however, the covalent probes have been nonspecific; *i.e.*, they react with several different amino acid side chains. This results in heterogeneous labeling and the subsequent measurements of fluorescent properties arise from, and are therefore averaged over, a number of different sites. This lack of specificity clearly limits the application and interpretation of the measurements with such probes.

We wish to report in this paper the synthesis and characterization of several new fluorescent reagents which combine the reactivity of iodoacetamide toward sulfhydryl groups with the spectral properties of the naphthalenesulfonic acids. The reagents are easily synthesized, water soluble, stable for long periods of time in crystalline form, and form stable conjugates with proteins. Like other naphthylaminesulfonic acids, they

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possess the following spectral properties: (a) invariance over a wide pH range, (b) stability in most solvents, (c) sensitivity to environment, (d) distinctness from spectral properties of protein chromophores, and (e) suitability of lifetimes for the determination of a useful range of rotational relaxation times. The resultant fluorescent reagents and their model compounds used for spectral characterization are given in Figure 1.

Experimental Procedures

Syntheses

p-Nitrophenyl Iodoacetate. Iodoacetic acid (3.72 g; 0.02 mol) and 3.34 g (0.024 mol) of *p*-nitrophenol were dissolved in 50 ml of ethyl acetate at 5°. To this was added, with stirring, 4.12 g (0.02 mol) of dicyclohexylcarbodiimide. The by-product, *N,N'*-dicyclohexylurea, started precipitating immediately. After complete dissolution of the dicyclohexylcarbodiimide, the temperature was held at 5° for 30 min with occasional stirring. The reaction mixture was allowed to attain room temperature and held there for 1 hr after which time it was filtered. The precipitate was washed with 50–100 ml of ethyl acetate and the combined filtrate and washings were flash evaporated at room temperature to dryness. The solid or oily residue was recrystallized from ethanol, the final crystals being washed several times with 10–15 ml of cold ethanol to yield light yellow-brown crystals. These were air-dried followed by drying *in vacuo* at 50°. A yield of approximately 50% was obtained in most preparations. The melting point was 74–77°, as compared to the literature value of 76° (Lorand *et al.*, 1962). *Anal.* Calcd for $C_8H_6INO_4$: C, 31.2; H, 1.96; N, 4.56; I, 41.4. Found: C, 31.6; H, 1.98; N, 4.74; I, 41.0.

N-(Aminoethyl)-5-naphthylamine-1-sulfonic Acid (1,5-EDANS) and the 1,8 Isomer (1,8-EDANS).[1] These compounds were synthesized in identical ways by the Bücherer reaction (Drake, 1942). Sodium 5-naphthol-1-sulfonate or sodium 8-naphthol-1-sulfonate (24 g; 0.1 mol) was added to 200 ml of an aqueous solution of 25% sodium bisulfite and stirred to maximum dissolution. Ethylenediamine (18 g; 0.3 mol) was added, the pH adjusted to 8 with concentrated HCl, and the mixture refluxed 12–24 hr. The desired product, a heavy precipitate, was visible within 1 hr with the 1,5 isomer, while the 1,8 isomer required a longer period. This conversion, in general, from a naphthol or naphtholsulfonic acid to the corresponding naphthylamine is easily followed by the red spectral shifts which occur in the longest wavelength absorption band (300–330 nm) and the fluorescence band (420–500 nm, blue to green). After cooling the reaction mixture to room temperature, the pH was adjusted to 7 with concentrated HCl, cooled to 5° for 1 hr, and filtered. The claylike residue was dissolved in water by the addition of sufficient 10 M NaOH and twice heated to boiling with activated charcoal and filtered. The resulting slightly yellow solution was cooled to room temperature, adjusted to pH 7 with HCl, and set at 5° for several hours. After filtration, the precipitate, which is the zwitterion of the sulfonic acid and alkylamine, was recrystallized twice from water and dried at 120° *in vacuo* for 24 hr yielding 11.9 g of the 1,5 isomer (45%) and 12.6 g of the 1,8 isomer (47%). *Anal.* Calcd for $C_{12}H_{14}N_2O_3S$: C, 54.2; H, 10.5; N, 5.26.

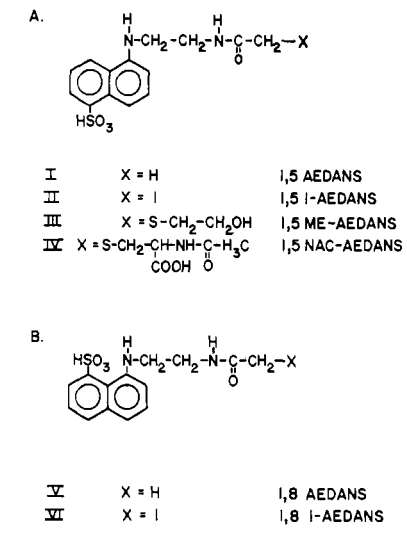

FIGURE 1: Fluorescent sulfhydryl reagents and model compounds. IV is referred to in the text as AcCys-AEDANS.

Found (1,5-EDANS): C, 54.04; H, 10.22; N, 5.33. Found (1,8-EDANS): C, 54.11; H, 9.6; N, 5.25.

N-(Iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic Acid (1,5-I-AEDANS) (II) and the 1,8 Isomer (1,8-I-AEDANS (VI). 1,5- and 1,8-EDANS were converted to their sodium salts by dissolving them in hot ethanol containing an excess of NaOH. Upon cooling, the salts (Na-EDANS) precipitated. These were then dried *in vacuo* at 80° and used without further characterization. The rest of the reaction steps were carried out in the dark due to the photosensitivity of the iodine-containing compounds. A mixture of 2.88 g (0.01 mol) of Na-EDANS and 12 ml of dimethylformamide at 5° was stirred to maximum dissolution. A solution of 4.6 g (0.015 mol) of *p*-nitrophenyl iodoacetate in 5 ml of dimethylformamide cooled to 5° was rapidly added to the Na-EDANS solution and allowed to react for 15 min. The mixture was then filtered rapidly. Two different routes were followed for the isomers: (a) for the 1,5 isomer, 1.62 ml of HI was added to the filtrate to ensure complete protonation of the aromatic amine; the addition of 40 ml of cold acetone and 10 ml of cold water with thorough mixing resulted in precipitation of the desired product (1,5-I-AEDANS) as the zwitterion; (b) for the 1,8 isomer 3.62 ml (0.02 mol) of HI was added to the filtrate, followed by 120 ml of cold water and 20 ml of cold acetone. Precipitation did not occur immediately, but was completed within 1 hr in the cold. After complete precipitation of both isomers, the solutions were filtered and the precipitates washed thoroughly with acetone and dried *in vacuo* at 80°. The yields were 3.2 g (95%) for the 1,5 isomer and 2.82 g (84%) for the 1,8 isomer. As these reagents are sensitive to light and moisture, they were stored in the dark *in vacuo*. *Anal.* Calcd for $C_{14}H_{15}N_2IO_3S$: C, 38.7; H, 3.46; N, 6.45; I, 29.2. Found (1,5-I-AEDANS): C, 38.93; H, 3.69; N, 6.59; I, 28.84. Found (1,8-I-AEDANS): C, 38.44; H, 3.42; N, 6.63; I, 29.01.

The extent of the iodoacetylation reaction and subsequent determination of reagent purity are easily determined by thin-layer chromatography. Aliquots of the reaction mixture or dye solution are diluted into 0.1 M phosphate buffer, pH 7, to which a small amount of β-mercaptoethanol has been added. The thiol compound reacts with the halogen-containing reagent giving the appropriate derivative. Another aliquot is treated with *N*-acetylcysteine under the same conditions. Migrations on silica gel thin-layer plates (Eastman Chemical

[1] Abbreviations used are: 1,5-EDANS, *N*-(aminoethyl)-5-naphthylamine-1-sulfonic acid; 1,8-EDANS, the 1,8-isomer; 1,5-I-AEDANS, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid; 1,8-I-AEDANS, the 1,8 isomer; 1,8-ANS, 1,8-anilinonaphthalenesulfonic acid.

TABLE I: R_F Values of Naphthylaminesulfonic Acid Derivatives on Silica Gel Plates with an Ethanol Eluent.[a]

Compound	R_F	Comments
1,5-EDANS	0.05	Starting material
1,5-I-AEDANS (II)	0.70	Product
1,5-I-AEDANS + β-mercaptoethanol	0.63	Similar to hydrolyzed product
1,5-I-AEDANS + *N*-acetylcysteine	0	Test for active halogen
1,8-EDANS	0.48	Starting material
1,8-I-AEDANS (VI)	0.82	Product
1,8-I-AEDANS + β-mercaptoethanol	0.77	Similar to hydrolyzed product
1,8-I-AEDANS + *N*-acetylcysteine	0	Test for active halogen

[a] See text for further details. Compounds are detected by illumination of the thin-layer chromatography plates with an ultraviolet lamp *after* chromatography is complete due to the photosensitivity of I-AEDANS.

Co.) with ethyl alcohol as the eluent give the results compiled in Table I.

N-(Acetylaminoethyl)-5-naphthylamine-1-sulfonic Acid (1,5-AEDANS) (I) and the 1,8 Isomer (1,8-AEDANS) (V). These compounds were prepared in the same way as the iodo analogs with the following modifications: (a) *p*-nitrophenyl acetate was used in place of the halo esters; (b) the reaction was performed in the light; (c) after the initial precipitation and acetone wash, the solid was dissolved in water, the pH adjusted to 7 with HCl, and the solution passed over a bed of carboxymethylcellulose on a Büchner funnel which had previously been equilibrated with 0.1 M sodium phosphate, pH 7. This removed a bluish fluorescent material which could not be removed by repeated precipitations. The pH of the filtrate was lowered to 2 with concentrated HCl and the solution set at 5° to complete precipitation. After filtering and washing with acetone, the residue was dried *in vacuo* at 80°. The yields were 60% for the 1,5 isomer and 38% for the 1,8 isomer. The 1,5 isomer was very hygroscopic and its analysis had to be corrected for water of hydration. *Anal.* Calcd for $C_{14}H_{16}N_2O_4S \cdot 0.5H_2O$: C, 53.0; H, 5.37; N, 8.83; S, 10.1. Found (1,5-AEDANS): C, 52.75; H, 5.60; N, 8.89; S, 10.47. *Anal.* Calcd for $C_{14}H_{16}N_2O_4S$: C, 54.6; H, 5.20; N, 9.1; S, 10.4. Found (1,8-AEDANS): C, 54.3; H, 5.21; N, 9.21.

Reaction of 1,5-I-AEDANS with β-Mercaptoethanol (1,5-ME-AEDANS) (III). A solution of 500 mg (1.15 mmol) of 1,5-I-AEDANS in water at pH 7 was mixed with a tenfold molar excess of β-mercaptoethanol, 0.67 ml, and the pH maintained at 7 for 1 hr by the addition of 1 M NaOH. The solution was adjusted to pH 2 with concentrated HCl and set in the cold. As little precipitation occurred after 24 hr, the solution was flash evaporated to dryness, and the residue was washed with ether and recrystallized from hot water. The crystals were dried *in vacuo* at 120°. *Anal.* Calcd for $C_{16}H_{20}N_2O_5S_2$: C, 50.0; H, 5.22; N, 7.29. Found: C, 49.66; H, 5.02; N, 7.48.

Reaction of 1,5-I-AEDANS with N-Acetylcysteine (1,5-AcCys-AEDANS) (IV). The reaction was conducted as above with the following quantities being used: 250 mg of 1,5-I-AEDANS, 1.05 g of *N*-acetylcysteine, and 4 ml of water. The recrystallized material was washed with 300 ml of acetone to remove the excess *N*-acetylcysteine and dried *in vacuo* at 120°. *Anal.* Calcd for $C_{19}H_{28}N_3O_7S_2$: C, 48.6; H, 4.91; N, 8.95; S, 13.65. Found: C, 48.31; H, 4.90; N, 8.95; S, 13.28.

Spectroscopic Determinations. Absorption spectra were automatically recorded on a Cary 15 spectrophotometer at 27° except where otherwise indicated.

Molar absorption coefficients were determined on a Zeiss PM QII spectrophotometer with a wavelength resolution of 2 nm. Absolute values were determined by dissolving weighed amounts of the desired sample, previously dried *in vacuo* at 80–110°, in 0.1 M phosphate buffer, pH 7.0 (reference solvent), and measuring the absorbance at the desired wavelengths. The molar absorption coefficients in other solvents were determined by diluting equal microliter aliquots of a concentrated stock solution of the dye in dimethylformamide into the desired solvents and the reference solvent and measuring the desired absorbances.

Fluorescence emission spectra were determined on the spectrofluorometer described by Weber and Young (1964) and modified to allow both right angle and front face illumination. The resolution for all spectra was 2 nm. Technical spectra were recorded on a time base recorder (Electronic 19, Honeywell, Inc.) without corrections for fluctuations in the exciting light intensity. Corrected spectra were determined as described by Scott *et al.* (1970).

Quantum yields were determined on the spectrofluorometer described above. When the relative yields of fluorophors with identical spectra were desired, the fluorescence intensities at one emission wavelength resulting from excitation of solutions of identical optical density at the excitation wavelength were measured. These intensities gave directly the relative quantum yields for the examined solutions. Comparison of fluorophors having nonidentical spectra was achieved by determining the corrected spectra as indicated above with the total emission being directly proportional to the quantum yields. Absolute quantum yields were determined by comparing the relative yield of a dye solution with that of a solution of equal optical density of quinine sulfate in 1 N H_2SO_4, assumed to have an absolute quantum yield of 0.70 (Scott *et al.*, 1970).

Polarization measurements were performed on the instrument described by Weber and Bablouzian (1966). Polarization excitation spectra were recorded point by point by varying the wavelength of excitation. Emission was usually selected by broad band-pass filters (Corning Glass Co.) and a 5 mm thickness of 2 M $NaNO_2$ to select for wavelengths of emission greater than 380 nm and to exclude the exciting light. Polarization emission spectra were recorded as above with the instrument modified to include a Jarrell-Ash (820410) grating monochromator inserted between the parallel polarizer and photomultiplier tube. Resolution of the emission was 3.3–6.6 nm. The reference photomultiplier emission was selected by filters as described above for excitation polarization.

Lifetimes were measured on the cross-correlation phase fluorometer described by Spencer and Weber (1969). The exciting light was sinusoidally modulated either at 28.4 or 14.2 MHz and passed through a Jarrell-Ash monochromator into the sample compartment, and the fluorescence selected by either a second identical monochromator or broad band-pass filters. The lifetimes were determined by phase and/or modulation measurements with resolutions of 6.6 nm on the excitation monochromator and 3.3–6.6 nm on the emission monochromator when filters were not used. The exciting light was polarized at 35° for measurements where energy transfer and rotational diffusion could produce anomolous results

TABLE II: Comparison of Spectral Properties of Derivatives of 1,5-AEDANS.[a]

Compound	λ_{abs}^{max} (nm)	λ_{em}^{max} (nm)	$10^{-3}\epsilon^{337}$ ($cm^2\ mM^{-1}$)	$10^{-3}\epsilon^{278}$ ($cm^2\ mM^{-1}$)
1,5-AEDANS	337	500	6.1	1.06
1,5-ME-AEDANS	337	498	5.8	1.00
1,5-AcCys-AEDANS	336	500	6.0	1.26

[a] λ_{abs}^{max} is the wavelength of maximum absorption of the last absorption band, λ_{em}^{max} is the wavelength of maximum emission, and ϵ^{337} and ϵ^{278} are the molar absorption coefficients at 337 and 278 nm, respectively. Resolution in all cases was ±1 nm.

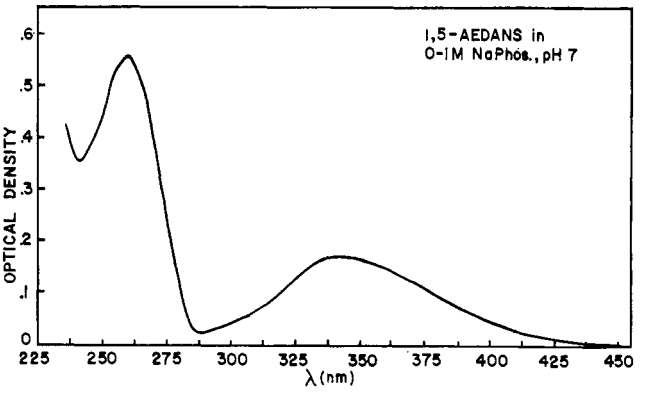

FIGURE 2: Absorption spectrum of 1,5-AEDANS in 0.1 M sodium phosphate, pH 7.0, 25°.

(Spencer and Weber, 1970). Temperature control to 0.1° was maintained throughout all experiments.

Miscellaneous Techniques. All titrations were carried out by transferring minute amounts of 1 M NaOH or 1 M HCl from a stock solution to the dye solution contained in an absorption or fluorescence cuvet.

The solubilities of the I-AEDANS isomers were determined by the following procedure. Saturated solutions of the dyes in buffers were prepared. Aliquots of these stock solutions were then diluted into buffer containing excess β-mercaptoethanol. The absorbances were then read at the appropriate maxima and the concentrations of the stock solutions calculated from the respective absorption coefficients.

Results

Properties of I-AEDANS. The 1,5 and 1,8 isomers of I-AEDANS undergo photodegradation in aqueous and organic solvent solutions and yield products which do not react with sulfhydryl compounds and have different spectral and chromatographic properties from both I-AEDANS and AEDANS. As AEDANS is not photosensitive, it is clear that iodine is responsible for the light reaction, a conclusion to be expected from previous studies on the photolability of alkyl halogens (Calvert and Pitts, 1966). The decomposition is complete within several minutes in dilute solutions upon irradiation with an ultraviolet hand lamp or monochromatic light from the xenon arc of the spectrofluorometer, but also occurs over longer periods when exposed to ordinary room lighting. Therefore, all preparations, reactions, purifications, and storage procedures involving the I-AEDANS isomers should be conducted with minimal exposure to light. The solubility of these dyes in phosphate buffer, pH 7, was determined to be at least 0.05 M. Samples of both isomers have been stored in brown bottles *in vacuo* for 3 years without any loss of reactivity.

Comparison of Three Analogs of AEDANS. The three model compounds, 1,5-AEDANS (I), 1,5-ME-AEDANS (III), and 1,5-AcCys-AEDANS (IV) were compared for several spectral properties and found to be quite similar (Table II). The simple analog, 1,5-AEDANS, was chosen for thorough spectral examination as the model compound to establish a set of reference properties for evaluating the environment of the fluorophore when covalently linked to macromolecules.

Absorption Spectra and Molar Absorption Coefficients of AEDANS. Figure 2 shows an absorption spectrum typical for the 1-naphthylaminesulfonic acids in a variety of solvents. All spectra exhibit maxima around 260 and 340 nm and a minimum around 280 nm. A summary of some spectral properties in various solvents and solvent mixtures is given in Tables III and IV. The longest wavelength absorption band is actually composed of at least two different electronic transitions as shown initially by Stryer (1965) and by Anderson and Weber (1969) for 1,8-anilinonaphthalenesulfonic acid (1,8-ANS) and verified in the present case by the polarization spectra to be presented later. There is also some variation in the absorption coefficients in the various solvents (Tables III and IV), although less than that reported by Turner and Brand (1968) for 1,7-anilinonaphthalenesulfonic acid (1,7-ANS).

Emission Spectra and Fluorescence Quantum Yields of AEDANS. Tables III and IV contain the emission maxima and quantum yields for the model compounds in a variety of solvents. The results are such as would be predicted from analogous studies with the naphthylaminesulfonic acids (Turner and Brand, 1968). In a given series such as ethanol–water, the emission maximum shifts continuously to the blue as the solvent polarity decreases, while the quantum yield in-

TABLE III: Absorption and Fluorescence Properties of 1,5-AEDANS in Various Solvent Systems.[a]

Solvent System	$\lambda_{abs}^{max\ b}$ (nm)	$10^{-3}\epsilon^{c}$ ($cm^2\ mM^{-1}$)	$\lambda_{em}^{max\ d}$ (nm)	Quantum Yield[e]
Water	336.0	6.1	520	0.27
Ethanol–water (20)[f]	337.0	6.3	510	0.40
Ethanol–water (40)	338.0	6.5	500	0.47
Ethanol–water (60)	338.5	6.6	493	0.56
Ethanol–water (80)	339.0	6.8	485	0.66
Ethanol	340.0	6.9	460	0.69
Propylene glycol	340.0	6.7	485	0.72
Dimethylformamide	341.0	8.1	448	0.82
Dioxane	339.0	7.4	452	

[a] All solutions were degassed with a stream of N_2 prior to fluorescence measurements. [b] The wavelength of maximum absorption in the longest wavelength absorption band. [c] The molar absorption coefficient at the absorption maximum with an error of 0.1. [d] The fluorescence maximum of the corrected emission spectrum. [e] Absolute quantum yields using quinine sulfate in 1 N H_2SO_4 as the standard of 0.70 with an estimated error of 10%. [f] The numbers in parentheses indicate the percentage of ethanol in the ethanol–water mixtures.

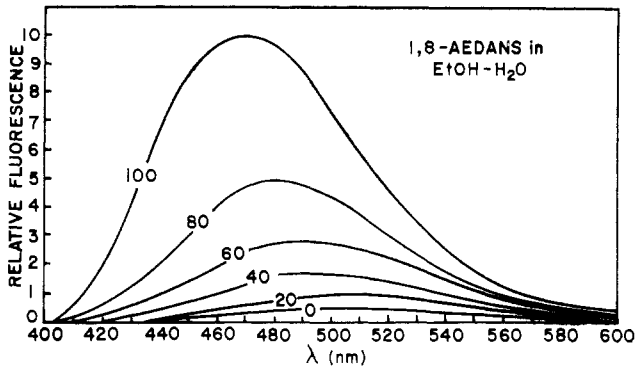

FIGURE 3: Emission spectra of 1,8-AEDANS in ethanol–water mixtures. Excitation was at the absorption maximum for each mixture with the numbers on the curves representing the per cent ethanol. All mixtures had equal concentration of dye equal to 3×10^{-5} M.

creases. This is shown in Figure 3 for 1,8-AEDANS in ethanol–water mixtures. Although the two isomers show the same general behavior, they differ in that 1,8-AEDANS is more sensitive to water quenching than the 1,5-AEDANS.

At room temperature, the emission maxima of both isomers in 1:2 propanediol are, as expected, independent of the excitation wavelength. However, at −55°, as shown in Table V, the emission maxima are dependent upon the excitation wavelength, similar to the dependency found in 1,8-ANS by Pasby (1969).

Fluorescence Lifetimes of AEDANS. The lifetimes of 1,5- and 1,8-AEDANS measured in a variety of solvents are shown in Table VI. As the solvent polarity decreases, the lifetimes and yields increase together (Tables III, IV, and VI) as the emission maxima shift to the blue. The 1,8-AEDANS shows the more dramatic change in lifetime and yield on comparing the aqueous and ethanolic solutions. The increase in lifetimes of 1,8-AEDANS in going from water to ethanol is particularly large with the maximum value being considerably greater than the maximum value for the lifetime of the 1,5-AEDANS.

The expected quenching by oxygen (Vaughan and Weber, 1970) proved to be quite significant in those systems with high concentrations of organic solvent. However, it should be noted that even 1,5-AEDANS was quenched slightly by oxygen in air-saturated water. The greater quenching in organic solvents as compared to water is due to the higher solubility of oxygen in the former at ordinary atmospheric pressure. It is important, therefore, to remove oxygen from

TABLE IV: Absorption and Fluorescence Properties of 1,8-AEDANS in Various Solvent Systems.

Solvent System	λ_{abs}^{max} (nm)	$10^{-3}\epsilon$ (cm^2 mM^{-1})	λ_{em}^{max} (nm)	Quantum Yield
Water	342.5	6.3	530	0.045
Ethanol–water (20)	345.5	6.8	520	0.073
Ethanol–water (40)	346.5	6.8	510	0.120
Ethanol–water (60)	347.5	7.3	500	0.19
Ethanol–water (80)	348.0	7.5	490	0.29
Ethanol	349.0	7.4	480	0.52
Propylene glycol	349.0	6.5	488	0.45
Dimethylformamide	352.0	7.6	463	0.71
Dioxane	352.0		480	

TABLE V: Dependence of Emission Maximum of 1,5- and 1,8-AEDANS in 1:2 Propanediol at −55° upon Excitation Wavelengths.

1,5-AEDANS		1,8-AEDANS	
λ_{ex} (nm)	λ_{em}^{max} (nm)	λ_{ex} (nm)	λ_{em}^{max} (nm)
330	444	320	449
350	442	350	450
380	445	380	450
390	448	400	454
400	450	410	457
410	453	430	460

model systems in order to properly evaluate the environmental effects upon quantum yields and lifetimes. Absorption and emission spectra, however, were found to be independent of oxygen concentration.

The lifetimes presented in Table VI were all taken with broad band emission and excitation at 340 nm. These conditions approximated those under which the quantum yields and polarization spectra were determined. Table VII summarizes the lifetimes as measured by phase and modulation at two excitation frequencies and at several wavelengths of emission for 1,5-AEDANS in phosphate buffer, pH 7.0. It is apparent from the 28.4-MHz lifetimes that heterogeneity does exist as indicated both by the discrepancy of the phase and modulation measurements and the different lifetimes at different emission wavelengths. This heterogeneity is not detected, however, at 14.2 MHz and is less apparent with the modulation measurements, both of which are less sensitive to shorter lifetimes than the 28.4-MHz phase measurements. Additional phase and modulation measurements at 14.2 MHz across the excitation band of 1,5-AEDANS in phosphate buffer and the emission bands of both 1,5-AEDANS and 1,8-AEDANS in ethanol revealed no heterogeneity with the lifetimes being 9.0 ± 0.2, 20.0 ± 0.3, and 25.3 ± 0.3, respectively. The heterogeneity is greatest at the blue end of the emission spectrum. Therefore, to maximize the probability of

TABLE VI: Lifetimes (Nanoseconds) of AEDANS Isomers in Air-Saturated and N_2-Flushed Solvents.[a]

Solvent	1,5-AEDANS		1,8-AEDANS	
	N_2	Air	N_2	Air
H_2O	9.4	8.9	2.7	2.0
Ethanol–H_2O (20)[b]	12.5	11.8	4.1	4.1
Ethanol–H_2O (40)	15.3	14.8	7.1	6.6
Ethanol–H_2O (60)	17.4	16.3	11.1	10.0
Ethanol–H_2O (80)	19.6	15.0	16.1	12.9
Ethanol	18.3	11.1	25.0	12.6
Propylene glycol	19.4		19.6	
0.1 M NaP_i, pH 7.0	9.1		3.1	
Dimethylformamide	16.1		25.8	

[a] Lifetimes were measured on the phase fluorometer at 28.4 MHz using modulation measurements; excitation was at 340 nm with emission viewed through the combination of a 2 M $NaNO_2$ filter and a 3-72 Corning filter. Error was estimated at ±0.2 nsec. [b] Number in parentheses indicates the per cent ethanol in ethanol–water mixtures.

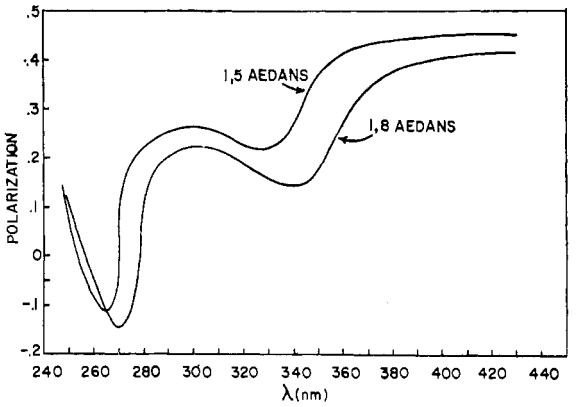

FIGURE 4: Excitation polarization spectra of 1,5-AEDANS and 1,8-AEDANS in 1:2 propanediol, −55°. Emission was viewed through 2 M NaNO and Corning 3-72 filters. Concentrations were 5×10^{-5} M.

homogeneous emission, it is recommended to confine measurements to the red edge of the emission band. In many cases, however, this may prove undesirable because of the large attenuation of the fluorescent signal.

Fluorescence Polarization Spectra of AEDANS. The excitation polarization spectra of 1,5- and 1,8-AEDANS in propylene glycol at −55° are given in Figure 4 and are similar in shape to those of other naphthylamines (Stryer, 1965) demonstrating the overlapping of at least two electronic transitions in the longest wavelength absorption band.

The emission polarization spectra of the two isomers were likewise determined under the same conditions as above with the results given in Figure 5. The varying polarization is indicative of heterogeneity across the band, in support of the lifetime and fluorescence spectra results, and again in agreement with the findings of Pasby for 1,8-ANS (Pasby, 1969).

pH Effects on AEDANS. Figure 6 shows the pH dependence of the lifetime, relative quantum yield, and optical density (335 nm) for 1,5-AEDANS in water. It can be seen that the general properties of AEDANS are consistent with those of other naphthylamines in which the following changes occur (Foerster and Renner, 1957; Lewis and Kasha, 1945): (a) at high pH (11–12) a proton is abstracted from the amino group of the *excited state* molecule resulting in quenching of the

TABLE VII: Dependence of Lifetimes of 1,5-AEDANS in Water upon Emission Wavelength and Modulation Frequency.[a]

λ_{em}	28.4 MHz		14.2 MHz	
(nm)	τ_m (nsec)	τ_p (nsec)	τ_m (nsec)	τ_p (nsec)
450			9.4	9.0
470	9.2	5.4	9.1	9.5
500	8.1	6.6	8.9	9.2
530	8.5	6.8	8.9	8.8
560	9.2	8.4	8.9	9.3
		Av	9.0 ± 0.2	9.2 ± 0.2

[a] Excitation is at 340 nm and emission resolution is ±3.3 nm. The solvent is 0.1 M sodium phosphate, pH 7.0. Estimated error is ±0.3 nsec. τ_p = lifetime measured by phase shift. τ_m = lifetime measured by degree of modulation.

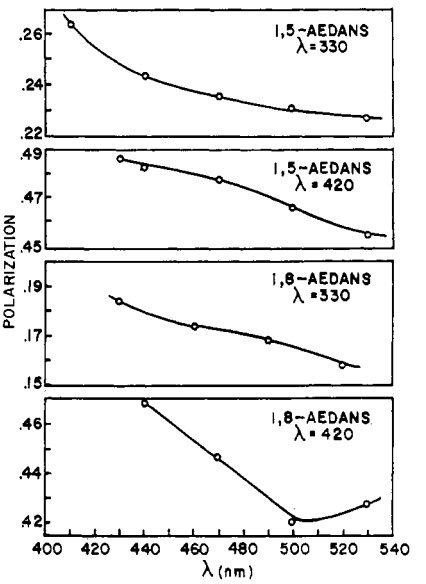

FIGURE 5: Emission polarization spectra of 1,5-AEDANS and 1,8-AEDANS in 1:2 propanediol at −55°. Each isomer was excited at two different wavelengths as represented on the spectra.

fluorescence and shortening of the lifetime; (b) at pH values below 4.0, protonation of the amino group eliminates the longest wavelength absorption band with a decrease in optical density, but no change in lifetime or quantum yield. There is a slight decrease in the quantum yield at low pH values, but the magnitude of the change is only 25% of that of the optical density change. Conversely, the parallel decrease in the lifetime and quantum yield at pH greater than 10 attests to the purely dynamic character of the alkali quenching. The significance of the constancy of the lifetimes in acid pH is that the fluorescence is simply proportional to the absorption at almost any wavelength and pH.

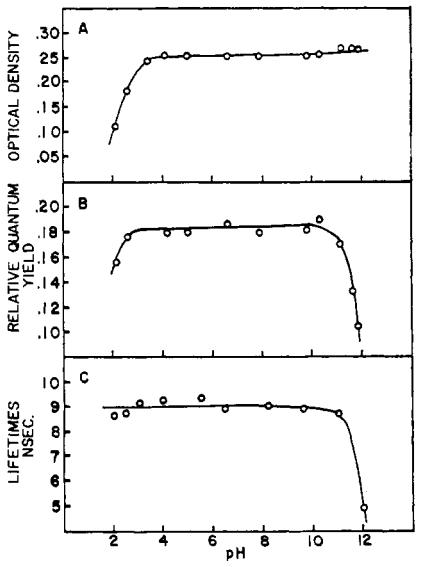

FIGURE 6: pH effects on the spectral properties of 1,5-AEDANS. (A) Optical density *vs.* pH, absorption wavelength 340 nm. (B) Relative quantum yield *vs.* pH with excitation wavelength 340 nm and emission wavelength 500 nm. Quantum yields were corrected for changes in optical density and geometry. (C) Lifetimes *vs.* pH with excitation wavelength 340 nm, emission selected by 2 M $NaNO_2$ and 3-72 Corning filters, and modulation frequency of 14.2 MHz.

Discussion

The sulfhydryl reagents synthesized, 1,5-I-AEDANS and 1,8-I-AEDANS, have the requisite properties for serving as useful fluorescence probes. They are easily prepared, highly purified, and can be stored dry in the dark for an indefinite period of time. The sulfonic acid substituent renders the reagents water soluble permitting all the advantages of ease of control and reproducibility of homogeneous reactions as opposed to the conditions of heterogeneous reaction of suspended reagents.

The spectroscopic properties of the reagents, as determined by studying the model compounds, 1,5-AEDANS and 1,8-AEDANS, are similar to those of other naphthylaminesulfonic acids (Turner and Brand, 1968), although the two isomers, themselves, differ significantly in spectral details. Their emission characteristics are highly dependent upon the solvent system, thus making them useful as "environmental probes." Conversely, the lack of any acid–base equilibria under normal conditions of measurement gives the emission, on the whole, a homogeneous character. This could be contrasted, for example, with the highly pH-dependent equilibria of other fluorophores such as fluorescein (Rozwadowski, 1961).

The reaction scheme developed for the synthesis of the AEDANS derivatives suggests the possibility of synthesizing a series of reagents composed of various combinations of the following three elements: (a) naphthylaminesulfonic acid isomer such as the 1,5, 1,8, or 1,4; (b) the alkyl chain, $(-CH_2)_n$, with different values for n; and (c) a reactive element terminal to the alkyl chain which could be, for example, iodoacetamido, maleimide, or carboxylsulfuric anhydride. The final result would be a series of dyes with similar spectroscopic properties and different reactive specificities, the latter of which could be determined primarily by the length of the alkyl chain and the nature of the reactive element terminating the chain.

The reagents, themselves, have been shown to react specifically with sulfhydryl groups in proteins such as globin and papain (Hudson, 1970), rhodopsin (Wu and Stryer, 1972), and myosin (Mendelson *et al.*, 1973). A following paper will deal specifically with conjugates of globin and papain.

A disadvantage of the I-AEDANS isomers is the photocatalyzed degradation. In addition to rendering the reactive species inactive, this degradation serves to contaminate conjugate solutions which have been "purified" by passage over an ion-exchange column which selects the negatively charged starting material but does not bind the degradation product (Hudson, 1970). In addition, a more serious difficulty is the possible reaction of the photoactivated excited state with various bonds in the protein in a manner similar to the reaction of diazo derivatives (Shafer *et al.*, 1966; Converse and Richards, 1969). Therefore, the specificity initially desired would be eliminated. To overcome these problems, all reactions and purifications involving these reagents should be conducted, if possible, in the dark.

Another problem to be noted is the heterogeneity observed in the emission spectra, polarization spectra, and lifetimes. The dependence of the emission upon the wavelength of excitation (Table V) amounts to a shift of some 5 nm as the excitation moves from the absorption maximum to a wavelength at which the absorption decreases to one-half maximum. Such a shift appears far too large to be due to any contaminating impurity and must therefore be considered intrinsic to the compound. Further evidence of heterogeneity in the emission is found in the observation of a conspicuous decrease in lifetime in the blue side of the emission spectrum when phase measurements are carried out at 28.4 MHz and also in the discrepancy between the phase and modulation measurements for this spectral emission region (Table VII). The fact that the lifetime observations refer to an aqueous solution at room temperature eliminates possible relaxation phenomena (Bakshiev, 1964; Ware *et al.*, 1968) as the cause of the discrepancy.

The nonuniform character of the emission polarization, shown in Figure 5, is observed to a smaller or larger extent in virtually every aromatic compound, and is particularly striking in the 1-naphthylamine derivatives like 1,8-ANS (Pasby, 1969). The causes are still obscure, but the generality of the phenomenon, as well as the dependence upon the wavelength of excitation in the present case, show that it results from the heterogeneous character of the emission, already revealed by a study of the emission spectrum and lifetime of the compounds.

This heterogeneity could conceivably arise from the existence of several configurations of the polarizable side chain attached to the ring nitrogen which differ in their relations with the naphthyl system. Since a similar heterogeneity is seen with other derivatives like 1,8-ANS and even 8-amino-1-naphthalenesulfonate (Pasby, 1969), it appears reasonable to suppose that an emission of degenerate character in the parent compound (1-naphthylamine) may be split to different degrees by substituents in the molecule or by interactions with the solvent. It is clear, however, that a thorough understanding of the origins of the heterogeneous character of the emission would help us to extract further information about the surroundings of the fluorophore and hence our interest in their clarification.

Acknowledgment

We wish to thank Dr. Richard Spencer for his helpful discussions and contribution to the fluorescent lifetime measurements.

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Quenching of Fluorescence by Oxygen. A Probe for Structural Fluctuations in Macromolecules†

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ABSTRACT: Quenching of the fluorescence of various fluorophores by molecular oxygen has been studied in aqueous and nonaqueous solutions equilibrated with oxygen pressures up to 100 atm. Temperature dependence of quenching, agreement with the Stern–Volmer equation, and fluorescence lifetime measurements indicate that essentially all the observed quenching is dynamic and close to the diffusion-controlled limits. Studies of charged polyamino acids containing tryptophan show that oxygen quenching, in contrast to I^-, is completely insensitive to charge effects. Ethidium bromide, when intercalated into double helical DNA, is quenched with 1/30th of the efficiency of the free dye in solution. Three dyes bound to bovine serum albumin were also found to be relatively protected from the free diffusion of oxygen. Quenching of intrinsic or bound fluorophores by molecular oxygen is therefore an appropriate method to determine the accessibility to oxygen of regions of the macromolecule surrounding the fluorophore and indirectly the structural fluctuations in the macromolecule that permit its diffusion to the fluorophore.

Molecular oxygen is known to be an efficient quencher of the fluorescence of aromatic hydrocarbons (Berlman, 1965; Ware, 1962). The studies so far published show quenching by oxygen to be a diffusion-controlled process in which virtually every collision with the excited fluorophore is effective in quenching. Although a good deal of work has been done in nonaqueous solutions, very little work has been done in aqueous solutions. This discrimination is due to the low solubility and diffusion coefficient of oxygen in water as compared to organic solvents, and the concomitant low levels of quenching that are observed in solutions equilibrated with air, or even with pure oxygen at atmospheric pressure.

Previous work in aqueous systems used pyrenebutyric acid (Vaughan and Weber, 1970).[1] Due to the long fluorescent lifetime of pyrenebutyric acid (100–200 nsec) it was possible to quench a significant fraction of its fluorescence using dissolved oxygen at 1 atm pressure. It was shown that when pyrenebutyric acid was bound to bovine serum albumin and other proteins the amount of quenching was greatly reduced. The protein structure prevented the diffusion of oxygen to the fluorophore.

Typical fluorescence probes have lifetimes of 10–20 nsec. Native tryptophan fluorescence of proteins displays lifetimes ranging from 2 to 6 nsec. Upon equilibration with oxygen at a pressure of 1 atm the fluorescence of a dye solution with a 2-nsec lifetime would decrease by only 3%; in a system where there is some steric barrier to the diffusion of oxygen to the fluorophore the decrease in signal would be even less. This problem may be overcome by increasing the oxygen concentration *via* an increase in the oxygen pressure. Equilibration of an aqueous solution with oxygen at a pressure of 100 atm results in a dissolved oxygen concentration (0.13 M) sufficient to quench more than one-half of the fluorescence of a dye with 1-nsec lifetime.

Since oxygen is an uncharged molecule of very small dimensions, and not particularly hydrophilic, as shown by its small solubility, the potential exists for yielding fundamentally different information from that obtained by the use of iodide, bromate, or mercuric ions as quenchers. Iodide quenching is known to be extremely sensitive to charge effects (Lehrer, 1971). In opposition to Hg^{2+}, oxygen at any of the concentrations used here does not form significant complexes with any of the dyes or proteins used in these studies. Hg^{2+} has a radius of action determined by the possibility of energy transfer from those fluorophores with emission spectra within the absorption band of the mercury–sulfur bond (Chen, 1971). In contradistinction, oxygen has no appreciable absorption at wavelengths longer than 250 nm, so that quenching requires actual short-range interaction with the fluorophore.

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[1] Barenboim (1963) reported the use of oxygen to quench photo- and radioluminescence of aromatic amino acids, proteins, and nucleic acids in solution and as dry powders. The use of polychromatic excitation (240–293 nm) comprising wavelengths of strong O_2 absorption, the smaller range of O_2 pressures (16 atm), the small number of proteins examined, and the absence of fluorescence lifetime measurements preclude comparison of his results with the present studies.