Fluorescent Probes for Conformational States of Proteins. I. Mechanism of Fluorescence of 2-*p*-Toluidinylnaphthalene-6-sulfonate, a Hydrophobic Probe*

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ABSTRACT: 2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS) is one of a class of compounds which do not fluoresce in water but fluoresce strongly both in organic solvents and when bound to certain native proteins. Because of the usefulness of TNS in studies of protein conformation, this compound was examined in a variety of solvents in an effort to analyze the mechanism of fluorescence. 2-Naphthylamine-6-sulfonate (NAS), a compound related to TNS, fluoresces both in water and in organic solvents. A comparison was made between TNS and NAS in order to define the contribution of the tolyl group to the mechanism of TNS fluorescence. The results indicate that TNS fluorescence is enhanced by solvents of low dielectric constant or, to a lesser extent, by solvents of high viscosity. These effects are interpreted in terms of a mechanism involving varying solvation of the excited state in solvents of different polarizability. On the basis of these and other studies we conclude that TNS is a probe for hydrophobic regions of proteins. This conclusion is supported by an examination of TNS fluorescence in solutions of proteins. Proteins which are known to possess hydrophobic binding sites (bovine serum albumin, chymotrypsin) promote high quantum yields of TNS fluorescence, whereas other proteins (lysozyme, γG-immunoglobulin, ovalbumin) promote much lower quantum yields. β-Lactoglobulin has also been found to promote high quantum yields of TNS fluorescence.

The fluorescence of proteins can be altered by changes in the local environment of their aromatic amino acid residues. This property of the intrinsic fluorescence of proteins can be used to study changes in their conformation (Steiner and Edelhoch, 1963; Gally and Edelman, 1962, 1965). Similarly, the fluorescence properties of organic molecules covalently linked to proteins may depend upon the conformational state of the protein (Weber, 1952b). Mechanistic interpretations of intrinsic fluorescent measurements are made difficult, however, by the absence of detailed information on the local environment of tryptophan and tyrosine residues. Compounds which are covalently bound may alter the protein and may label different molecules of the same protein in a variable and unknown fashion. Moreover, the sites to which covalently bound molecules are attached may not coincide with those regions of the protein molecule which are important in maintaining its conformation.

In the present report, we describe the properties of a fluorescent compound which may be used as a probe of protein conformation in methods which avoid some of the aforementioned limitations. Fluorescent probes may be defined as small molecules which undergo changes in one or more of their fluorescent properties as a result of noncovalent interaction with a protein or other macromolecule. Fluorescent probes are analogous to adsorption indicators (Laurence, 1952) and their behavior may be studied by standard equilibrium methods. Two examples of fluorescent probes are 1-anilinonaphthalene-8-sulfonate[1] and 2-*p*-toluidinylnaphthalene-6-sulfonate. These compounds are practically nonfluorescent in aqueous solutions, but fluoresce strongly when adsorbed to bovine serum albumin and to denatured proteins (Weber and Laurence, 1954). Fildes *et al.* (1954) proposed a fluorometric method which employs ANS for the detection of albumin in serum. Newton (1954a,b) used an isomer of TNS to study the action of polymyxin on *Pseudomonas aeruginosa*. The interaction of TNS with antibodies has been examined by Winkler (1962). More recently, Weber and Young (1964) have used ANS to study the acid expansion of bovine serum albumin. Gally and Edelman (1965) found that changes in ANS fluorescence accompany the thermal transitions of Bence–Jones proteins. The binding of ANS in the heme crevice of apomyoglobin and apohemoglobin has been investigated

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[1] Abbreviations used: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; ANS, 1-anilinonaphthalene-8-sulfonate; NAS, 2-naphthylamine-6-sulfonate; ω, wavenumber in cm^{-1}. In order to avoid confusion with extinction coefficients, D is used instead of ϵ for the dielectric constant.

by Stryer (1965). Alexander and Edelman (1965), in a preliminary communication, described the use of TNS as a probe of protein conformation.

Ideally, the changes in fluorescence of a probe should be interpretable in terms of its interaction with particular structures on the protein. The investigations to be described below provide evidence that TNS is a probe for hydrophobic regions in proteins. We have found that TNS fluorescence is enhanced in solvents of low dielectric constant or of high viscosity. The high quantum yields characteristic of TNS dissolved in protein solutions must be due in large part to interactions between TNS and hydrophobic regions on the protein surface.

Materials and Methods

TNS was prepared using a modification of the method proposed by Houben and Weyl (1957) for the synthesis of 1-anilinonaphthalene-8-sulfonic acid. In a typical preparation of TNS, 1.44 g (0.01 mole) of toluidine hydrochloride (Matheson Coleman and Bell, East Rutherford, N. J.), 10.7 g (0.1 mole) of toluidine (Matheson Coleman and Bell, East Rutherford, N. J.), and 4.46 g (0.02 mole) of 2-aminonaphthalene-6-sulfonic acid (K and K Laboratories, Plainview, N. Y.) were ground together in a mortar and heated to 150° in a small flask. The flask was lightly stoppered to prevent loss of toluidine and maintained at 150° for 20–24 hr, at which time the end of the reaction was indicated by the complete solubility in methanol of a small aliquot of the reaction mixture. The product was cooled, thoroughly triturated with 200 ml of 1 M HCl, and filtered. The silver gray crystals were washed with 1 l. of 1 M HCL and with two aliquots of 25 ml of acetone. The washed crystals were dissolved in 250 ml of gently boiling water which contained 0.5 g of KOH. After decolorization with 1.0 g of Norit, the solution was stored overnight at 4° and filtered. The white product was recrystallized twice more (using 100 ml of water for each crystallization), rinsed, and dried on the funnel with a small amount of acetone. Yields varied from 30 to 60%.

Anal. Calcd for $C_{17}H_{14}KNO_3S$: C, 58.07; H, 4.01; K, 11.12; N, 3.99. Found: C, 58.10; H, 3.97; K (from ash), 11.17; N, 3.99.

The potassium salt could be converted to an acidic derivative without difficulty. To a solution of 1.0 g of the salt in 50 ml of hot water was added 5 ml of 1.0 M HCl. After chilling at 4° for *ca.* 6 hr, white lustrous plates were collected by filtration and dried *in vacuo* over P_2O_5 at room temperature.

Both the potassium salt and the acidic derivative of TNS melt with decomposition. In order to obtain a derivative possessing a distinctive melting point the dicyclohexylammonium salt was synthesized. The acidic derivative (250 mg) was dissolved in 5 ml of 80% *n*-propyl alcohol, and 1 ml of redistilled dicyclohexylamine (J. T. Baker Chemical Co., Phillipsburg, N. J.) was added to the solution. After crystallization was initiated by scratching the walls of the vessel, 20 ml of diethyl ether was added and the solution stored for 60 min. The white crystals of the dicyclohexylammonium salt were collected, washed with ether, and stored in the dark; yield, 92%; melting range, 238–239° (capillary tube, uncorrected).

During all of the synthetic procedures and subsequent handling of TNS and its derivatives, attempts were made to minimize exposure to light from fluorescent fixtures in the room. No change in the properties of these compounds was detected after 1–2-hr exposures to room lighting, but 24- or 48-hr exposures led to the formation of several fluorescent products. These contaminants can be demonstrated near the origin of ascending thin layer chromatograms developed with isobutyl alcohol saturated with 3% aqueous ammonia.

TNS synthesized by the aforementioned procedure was examined for heterogeneity by ascending thin layer chromatography (Eastman Chromagram K301R, Rochester, N. Y.) utilizing over 40 different solvent systems (R. S. Schwyzer, personal communication, 1964; Schwyzer and Sieber, 1966). In all systems the material moved as a single spot with no evidence of heterogeneity. As a further criterion of purity, the emission spectrum of TNS was studied at different wavelengths of excitation as suggested by Weber (1961). The emission spectrum was independent of the excitation wavelength from the long wavelength limit of the excitation spectrum (*ca.* 380 mμ) to the shortest wavelength which could be used with available light sources (*ca.* 280 mμ), in solvents which promote high quantum yields (ethyl alcohol, *n*-butyl alcohol) and low quantum yields (formamide, ethylene glycol).

Extinction coefficients for TNS in water were obtained from the slope of plots relating absorbance and concentration by weight. No deviations from the Beer–Lambert law were observed at concentrations up to 10^{-4} M.

Throughout this work, reagent grade chemicals of the highest available purity were obtained from the quoted sources. Unless otherwise specified, solvents used in the syntheses and spectral measurements were distilled through a 120-cm fractionating column packed with Raschig rings and equipped with a silvered vacuum jacket. Solvents with boiling points in excess of 150° (760 mm) were purified by vacuum distillation using the same column. Most of the solvents were characterized by boiling range, boiling point, and refractive index (n_D^{20}). Solvents were used only if they possessed (a) boiling ranges of 0.2–0.3° and boiling points which agreed with literature values (Weissberger *et al.*, 1955) to within the calibration error of the head thermometer; and (b) refractive indices (Abbé refractometer) which varied from literature values (Weissberger *et al.*, 1955) by $< \pm 0.0008$.

Ficoll (Pharmacia Chemicals, Inc., New York, N. Y.; lot no. 4624) was dried for several days over P_2O_5 *in vacuo* before being used to prepare solutions. Solutions of Ficoll and sucrose were prepared by weight, as were mixtures of these solutions. The stock solution of 20% Ficoll was filtered through a fine-sintered glass filter before dilution with 20% sucrose. Viscosities of Ficoll–

TABLE I: Molecular Weights and Extinction Coefficients.

Protein	Mol Wt	E^a	Ref
γG-Immunoglobulin	160,000	1.45[b]	Oncley *et al.* (1949)
Bovine plasma albumin	69,000	0.667[b]	Foster and Sterman (1956)
Lysozyme	14,100	2.63	Sophianopoulos *et al.* (1962)
β-Lactoglobulin	35,500	0.95	Boyer (1954)
Urease	483,000	...	Sumner *et al.* (1938)
Ovalbumin	45,000	0.735	Cunningham and Nuenke (1959)
Trypsin	23,800	1.44	Davie and Neurath (1955)
Trypsinogen	23,800	1.39	Davie and Neurath (1955)
α-Chymotrypsin	25,000	2.06[c]	Dreyer *et al.* (1955)
Chymotrypsinogen A	25,000	2.06[c]	Schwert and Kaufman (1951)
Pepsin	35,500	1.43	Edelhoch (1957)
Pepsinogen	41,000	1.25	Arnon and Perlmann (1963)

[a] Absorbance observed at 1 mg/ml, 1-cm path length at 280 mμ unless otherwise specified. [b] 278 mμ. [c] 282 mμ.

sucrose solutions were obtained with a Cannon–Fenske capillary viscometer (size 100). Densities of the solutions were obtained using a pycnometer of 5-ml nominal volume, calibrated with mercury. Both residence times and densities were obtained at 25.0°, using a bath which was regulated to ±0.01° (Beckmann thermometer). Refractive indices were measured with an Abbé refractometer at 25.0 ± 0.1°.

The buffers used in studies of proteins had an ionic strength of 0.1 M and had the following pH and composition: pH 2.3, KCl–HCl; pH 5.0, NaOH–acetic acid; pH 7.4, NaOH–KH_2PO_4; pH 8.0, Tris–HCl. The proteins used were obtained commercially from the following sources: from Armour Pharmaceutical Co., Kankakee, Ill.: bovine serum albumin, lot U68712; bovine γ-globulin (fraction II from bovine plasma), lot A30702; from Nutritional Biochemicals Corp., Cleveland, Ohio: urease, lot 3422; from Pentex, Inc., Kankakee, Ill.: β-lactoglobulin, crystallized, lot 32; ovalbumin, five times crystallized, lot 10; from Worthington Biochemical Corp., Freehold, N. J.: α-chymotrypsin I, lot CDI 6112-5, and chymotrypsinogen A, lot CG 763, both three times crystallized; lysozyme, twice crystallized, salt free, lot LYSF 638; pepsin, twice crystallized, lot 661; pepsinogen, crystalline, lots PG 114, PG 9163; trypsin, twice crystallized, lyophilized, salt free, lot 6223; trypsinogen, once crystallized, lot 6402.

Solutions of protein for use in fluorescence measurements were made up by weight in appropriate buffers (Table III) and were diluted, when possible, to yield a final concentration of 10 mg/ml. Concentrations were based on the absorbance of samples diluted to a final maximal absorbance of 0.4–0.6. Molecular weights and extinction coefficients used in calculations involving proteins are given in Table I.

Corrected emission spectra were recorded using the instrument described by Rosen and Edelman (1965). Excitation spectra obtained with the same instrument were corrected to constant incident quanta using a Rhodamine B quantum counter (0.8 mg/ml in freshly distilled ethylene glycol) as described by Melhuish (1962). Measurements of polarization of fluorescence (Weber, 1952a) were carried out with this machine by obtaining $I_\parallel$ and $I_\perp$ and calculating the polarization p:

$$p = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp} \quad (1)$$

In measurements of the fluorescence polarization of TNS in glycerol, the emission was isolated with a Corning 0-51 filter. No corrections were applied to the measured polarizations. In order to examine the validity of this method of measurement the fluorescence polarization of Rhodamine B in glycerol was measured at 25°. Within the wavelength range of 480–540 mμ the polarization remained constant at 0.444 ± 0.002, in agreement with published values of 0.444 (Weber, 1956) and 0.445 (D. A. Deranleau, 1965, personal communication).

In all fluorescence measurements the exciting monochromator was operated with slit widths of 2 mm (6.7 mμ at half-peak height) and the emission monochromator was operated at slit widths of 6 mm (10.0 mμ at half-peak height). Absorbance spectra were measured using a Cary Model 14 spectrophotometer (Applied Physics Corp., Monrovia, Calif.) equipped with a 0–0.1 absorbance unit slidewire. Slit widths of 0.1–0.3 mm were not exceeded in routine measurements.

Temperature control was maintained by circulating water from a thermostat through a copper block surrounding the cell. Direct measurements of temperature stability were made with a thermistor probe. Unless otherwise stated, all measurements were carried out at 25.0 ± 0.1°. Oxygen was removed from nonaqueous solvents by bubbling helium through the solution for 8–15 min immediately before measurements of fluorescence.

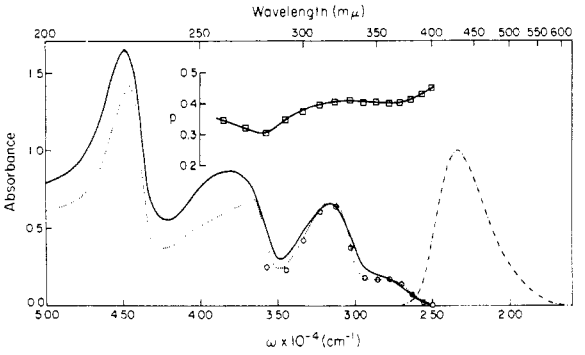

FIGURE 1: Spectra of TNS in various solvents at 25°. The following spectra were obtained: absorbance spectrum in water (——); absorbance spectrum in ethyl alcohol (-----); excitation spectrum in ethyl alcohol (—○—); emission spectrum in ethyl alcohol (— - —); and polarization spectrum in glycerol (□). The emission spectrum has been normalized to 1.0 at the wavelength of maximal emission. ω, wavenumber; p, polarization. Concentrations of TNS: absorbance spectrum in water, 3.5×10^{-5} M; absorbance spectrum in ethyl alcohol, 2.6×10^{-5} M; emission and excitation spectra, 8.3×10^{-6} M; and polarization spectrum, 4.6×10^{-6} M.

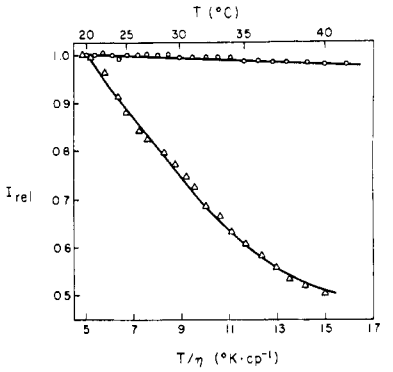

FIGURE 2: The variation of fluorescent intensity with T/η for NAS (○) and TNS (Δ) in 60% sucrose. Intensities were expressed as the ratio of the intensity at temperature T to the intensity at 20.0° (I_{rel}). Data relating the temperature T and the viscosity η were taken from Othmer and Silvis (1948). Wavelengths of excitation: NAS, 355 mμ; TNS, 366 mμ. Analyzing wavelengths: NAS, 415 mμ; TNS, 486 mμ.

The quantum yield (ϕ) of a sample was calculated from the observed absorbance (A) and the area enclosed by the emission spectrum using the method of Parker and Rees (1960)

$$\phi_s = \phi_q \frac{(1 - 10^{-A_q})}{(\text{Area})_q} \frac{(\text{Area})_s}{(1 - 10^{-A_s})} \qquad (2)$$

when the subscripts s and q refer to measurements made with the sample and a quinine sulfate reference, respectively. The quantum yield of quinine sulfate (*ca.* 10^{-5} M) in 0.5 N H_2SO_4 was taken as 0.55 (Melhuish, 1961). When measured using this method, the quantum yield of fluorescein in 0.1 M NaOH was 0.83, in agreement with literature values (Parker and Rees, 1960). Absorbancies were measured at the wavelength of excitation, which was 366 mμ for TNS and 355 mμ for NAS. Concentrations of TNS and NAS were maintained between 5×10^{-6} and 20×10^{-6} M, corresponding to absorbances of 0.07 or less at the wavelengths of excitation. Quinine sulfate solutions were also restricted to absorbances of <0.06. No dependence of the quantum yield upon concentration was noticed within these limits.

Integrated areas of emission spectra were measured either by planimetry or by weighing the paper under the peak. In either case measurements were referred to a known area of chart paper immediately adjacent to the curve of interest. The two methods gave results which agreed to within ±0.5%.

Effective band widths in mμ were evaluated by dividing the area of a given emission spectrum by the peak height. Band widths were converted from wavelengths to wavenumbers by

$$\Delta\omega = \frac{\Delta\lambda}{\lambda_{max}^2} \times 10^7 \qquad (3)$$

where $\Delta\omega$ and $\Delta\lambda$ are the band widths in cm^{-1} and mμ, respectively, and λ_{max} is the wavelength of maximal emission. Equation 3 was checked in several solvents of widely varying band widths and emission maxima by converting the recorded spectra directly from wavelengths to wavenumbers and determining $\Delta\omega$ from the measured area and height of the peak. The directly determined and calculated values of $\Delta\omega$ were within 1–2% of each other.

Results

The absorbance spectrum of TNS in water is given in Figure 1. In agreement with spectra of other positional isomers of the *N*-arylaminonaphthalenesulfonates, the absorbance spectrum of TNS possesses bands centered at 44,820 cm^{-1} [223 mμ (ϵ 4.70×10^4)] and 38,020 cm^{-1} [263 mμ (ϵ 2.45×10^4)] and the broad, less intense amine band at 28600–27800 cm^{-1} [366 mμ (ϵ 4.08×10^3)]. In addition to these bands, TNS absorbance spectra show the presence of a peak at 31,560 cm^{-1} [317 mμ (ϵ 1.89×10^4)] which is characteristic of 2-substitute daminonaphthalene derivatives (Hirshberg and Jones, 1949).

Preliminary experiments were conducted to determine the effect of varying pH upon the TNS absorbance spectrum in water. The spectrum of Figure 1 was invariant at all pH values between 2 and 14, although solutions of greater acidity suppressed the absorbance at all wavelengths.

TNS is not fluorescent in water, but fluoresces

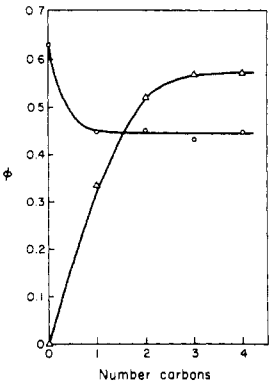

FIGURE 3: The variation of the quantum yield ϕ of NAS (O) and TNS (Δ) in primary aliphatic alcohols of increasing chain length. Values obtained in water are plotted with an abscissa of 0.

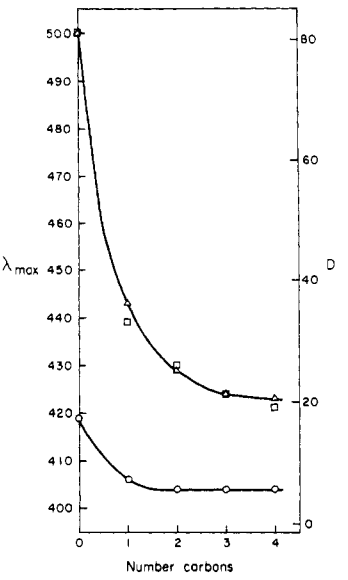

FIGURE 4: The variation of the wavelength of maximal emission (λ_{max}) for NAS (O) and TNS (Δ) dissolved in primary aliphatic alcohols of increasing chain length. Squares ($\square$) refer to the static dielectric constants (D) of the same alcohols (Slevogt, 1939).

strongly in ethyl alcohol. In order to compare the two solvents, the absorbance spectrum of TNS in ethyl alcohol is given in Figure 1. As compared to the spectrum in water, the spectrum in ethanol had sharper peaks which were slightly shifted to longer wavelengths. These effects are particularly evident in the band centered near 260 mμ. They were observed in all solvents in which TNS gave a significant quantum yield of fluorescence.

Figure 1 also contains the corrected excitation and emission spectra of TNS in ethyl alcohol. The excitation spectrum has been normalized to the absorbance spectrum at 360 mμ. The agreement between the absorbance spectrum and the corrected excitation spectrum is within a reasonable error at wavelengths as low as 290 mμ, below which diminished light source intensities made comparison difficult because of the very large corrections required. The 320-mμ band of the absorbance spectrum is clearly evident in the excitation spectrum.

The corrected emission spectrum is given on the right-hand side of Figure 1. There is little overlap of absorbance and emission spectra. The emission spectrum was independent of the exciting wavelength within the range examined (290–380 mμ).

TNS has little or no observable fluorescence when dissolved in water. It was noticed, however, that solutions of TNS in 60% sucrose exhibited a marked fluorescence. Moreover, aqueous solutions of TNS fluoresce when frozen and all the derivatives of TNS which we have prepared possess a brilliant blue fluorescence in the solid state.

TNS fluoresces in glycerol. The fluorescence polarization spectrum of TNS in glycerol was obtained at 25° using exciting radiation which varied from 260 to 400 mμ (Figure 1). The polarization was approximately constant from 310 to 370 mμ, but showed a definite change at lower wavelengths. At 366 mμ, the wavelength of excitation used for quantum yield measurements, the polarization had a value of 0.403.

In sharp contrast to TNS, its synthetic precursor NAS yields a bright blue fluorescence when dissolved in water. To investigate the influence of the tolyl substituent, the effect of temperature on the fluorescence of NAS and TNS was studied. Solutions of either NAS or TNS in 60% sucrose were heated from *ca.* 18 to >40° while continuously monitoring their fluorescence, using exciting and analyzing wavelengths which yielded maximal intensities at the lowest temperature studied. The results of these experiments are given (Figure 2) in terms of the observed temperature (T) and viscosity (η). Although NAS showed very little decrease in the intensity of fluorescence with increasing temperature, the intensity of TNS fluorescence decreased by 50% over the temperature range of 20–40°.

A second difference in the behavior of NAS and TNS was found during measurements of the quantum yields of fluorescence in water and in straight-chain aliphatic alcohols ranging in carbon chain length from C_1 to C_4 (Figure 3). The fluorescence of NAS in water decreased to a constant value in the alcohols, whereas the intensity of TNS fluorescence progressively increased with increasing chain length of the alcohols. Stryer (1965) has observed a similar shift of λ_{max} for ANS in these solvents. During these experiments a marked correlation between decreasing λ_{max} and decreasing dielectric constant was noticed. Data which demonstrate this point in the case of TNS have been included in Figure 4, where the dielectric constant scale has been adjusted to superimpose the dielectric constant and λ_{max} in water and *n*-propyl alcohol. The correlation is seen to be quite good.

The emission spectra of NAS and TNS may be compared by means of the wavelength of maximal emission (λ_{max}). In Figure 4, data are presented which relate λ_{max} for NAS and TNS in water and alcohols of varying chain length. Although the λ_{max} values of both fluorescent compounds decreased with increases in the length of the carbon chain of the alcohols, the effect was much more pronounced with TNS.

TABLE II: Quantum Yields and Related Parameters of TNS Fluorescence in Selected Solvents.[a]

Solvent	Quantum Yield (ϕ)	Emission Max λ_{max} (mμ)	Emission Max ω_{max} ($cm^{-1} \times 10^{-4}$)	Peak Width[b] ($\Delta\omega$) (cm^{-1})
Alcohols				
Methyl	0.34	443	2.24	5140
Ethyl	0.52	429	2.33	4410
n-Propyl	0.57	424	2.36	4160
Isopropyl	0.53	421	2.38	4080
n-Butyl	0.57	423	2.36	4360
Isobutyl	0.54	421	2.38	4270
sec-Butyl	0.50	423	2.36	4270
t-Butyl	0.53	425	2.35	4240
Allyl	0.49	432	2.32	4520
Acids				
Acetic	0.18	444	2.25	5240
Propionic	0.23	431	2.32	4540
n-Butyric	0.25	425	2.35	4160
Isobutyric	0.22	425	2.35	4160
Miscellaneous				
Water	0.0008	500	2.00	4850
Ethylene glycol	0.14	456	2.15	5730
Glycerol[c]	0.24	465	2.15	5360
20% Sucrose	0.005	500	2.00	5390
60% Sucrose	0.015	486	2.06	5130
20% Ficoll	0.085	465	2.15	5120
Formamide	0.032	487	2.05	5320
N-Methylformamide[c]	0.14	465	2.15	5400
Dimethylformamide	0.54	427	2.34	4050
Acetone	0.12	418	2.39	3920
Acetic anhydride	0.22	427	2.34	4550
Ethyl acetate	0.42	418	2.39	3950
Acetonitrile	0.49	428	2.34	4220
Diethylamine	0.022	416	2.40	4080
Pyridine	0.002	420	2.38	4040
90% Phenol	0.065	480	2.08	5520
Dioxane	0.30	413	2.42	3700
90% Dioxane	0.29	434	2.30	4720
80% Dioxane	0.28	438	2.28	4940
70% Dioxane	0.25	443	2.26	5060
60% Dioxane	0.17	454	2.20	5480
50% Dioxane	0.10	465	2.15	5670
40% Dioxane	0.057	473	2.11	5820
30% Dioxane	0.024	486	2.06	5440
Dioxane–$SnCl_2$[d]	0.44	441	2.27	5050

[a] Excitation at 366 mμ, 25°. [b] Peak area divided by the peak height. See text for details. [c] Not redistilled. [d] Refluxed and distilled from $SnCl_2$.

In order to gain insight into the nature and the mechanism of TNS fluorescence, measurements of the quantum yield, effective band width, and λ_{max} were carried out in a variety of solvents (Table II). The straight-chain aliphatic alcohols were examined first, with the results given above. It is apparent that within a homologous solvent series an increase in the quantum yield is accompanied both by a decrease in λ_{max} and by sharpening of the emission band. Comparisons of the fluorescence properties of TNS in the isomers of propyl and butyl alcohols indicate that branching of the carbon skeleton caused small, but probably real, decreases in the quantum yield. The presence of unsaturation, as found in allyl alcohol, resulted in a significant decrease in the quantum yield as compared to *n*-propyl alcohol.

Also included in Table II are data obtained for the fluorescence of TNS in short-chain fatty acids. These data provide further evidence for the correlation of an increase in the quantum yield, a decrease in the peak width, and an increasing number of methyl or methylene groups in the solvent molecule. This correlation is quite apparent within any given homologous series, although the correlation between quantum yield and either λ_{max} or the number of methylene groups becomes much poorer as comparisons are made between any two nonhomologous solvents. Preliminary experiments using fatty acids as solvents for TNS had indicated that TNS was not fluorescent in formic acid; however, this has been found to be due to protonation of the TNS. The form of TNS which is present in formic acid has much less absorbance than TNS in water, but possesses an absorbance spectrum identical with that of TNS in 1 M HCl. The fluorescent properties of this protonated species differ from those of TNS in the solvents in Table II. Emission spectra of TNS in formic acid had maxima at 328 and 340 mμ, and were identical using excitation wavelengths of 287, 305, and 315 mμ.

Solutions of TNS in formamide and the two methylated derivatives of formamide were also studied. The same correlations noted for the alcohols and acids were observed in this series of solvents.

The remaining solvents of Table II were studied to determine the effects of some specific chemical groupings on TNS fluorescence. It is apparent from the data obtained in solvents such as acetone and the amines that a short wavelength emission is not always accompanied by a high quantum yield. In general, those solvents in which TNS possesses both a low quantum yield and a low λ_{max} possess either nitrogen atoms or keto groups. Although emission spectra having low λ_{max} values may or may not be accompanied by a high quantum yield, solvents which yielded broad bands with maxima at longer wavelength invariably gave low quantum yields of TNS fluorescence.

The insolubility of TNS in several interesting solvents

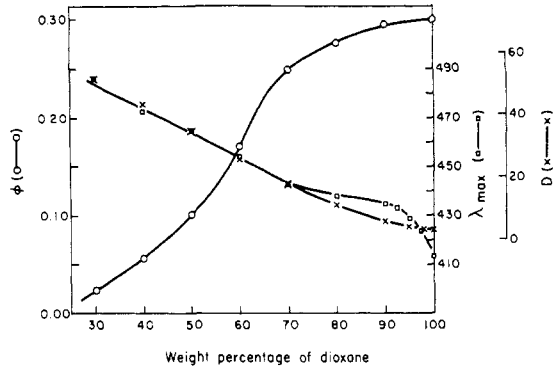

FIGURE 5: The variation of quantum yield, ϕ (O) and the wavelength of maximal emission, λ_{max} (□) of TNS fluorescence in dioxane–water mixtures. For comparison, the dielectric constants, D (x) of dioxane–water mixtures (Åkerlöf and Short, 1936) have been superimposed upon the λ_{max} data. Data were collected at 25.0° using 366-mμ excitation. The ordinate for dielectric constant values is at the extreme right.

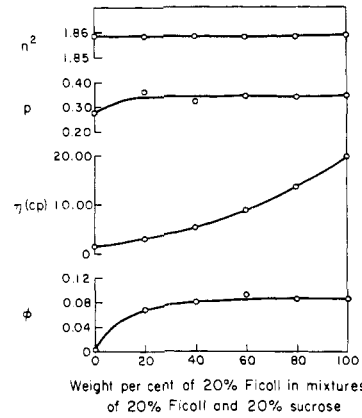

FIGURE 6: Correlation of quantum yield ϕ of TNS fluorescence with viscosity η of sucrose–Ficoll mixtures. p, polarization; n^2, square of the refractive index at 25.0°, sodium D line.

prevented their inclusion in Table II. All of these solvents are paraffinic (petroleum ether (bp 60–70°), hexane, cyclohexane), aromatic (benzene), or else possess large numbers of paraffinic chains (triethylamine).

Solutions of TNS in dioxane had the shortest wavelength emission of any system examined. In order to explore further the relation between various properties of TNS emission spectra and the dielectric constant of the solvent, TNS fluorescence was examined in aqueous solutions of dioxane. In each solution the quantum yield and λ_{max} were measured as a function of the weight percentage of dioxane (Figure 5). The band widths in each solution were also obtained and are presented in Table II. Dielectric constants of dioxane–water mixtures have been included in Figure 5 by superimposing the values of λ_{max} and D at 30 and 70% dioxane. An increasing quantum yield was again associated with a decreasing λ_{max} (and a narrowing of the peak). The λ_{max} values observed for TNS fluorescence in dioxane–water mixtures were closely correlated with the static dielectric constant, particularly in the range between 30 and 70% dioxane. At higher dioxane concentrations both the dielectric constant and λ_{max} undergo anomalous changes, with a fairly sharp decrease in λ_{max} between 90 and 100% dioxane. The observed value of λ_{max} in 100% dioxane agrees well with the value obtained by extrapolating the linear portion of the λ_{max} curve. Sharp changes in several thermodynamic properties (*i.e.*, heat of vaporization) of dioxane–water mixtures have been observed in the range of very low water concentration (Stallard and Amis, 1952).

The results of heating TNS and NAS in 60% sucrose (Figure 2) suggest that, in addition to changes in the dielectric constant, changes in either the temperature and/or solvent viscosity can affect TNS fluorescence.

In order to examine alone the effect of varying viscosity upon the fluorescence of TNS, the following experiment was carried out. A stock solution of 20% Ficoll was prepared and diluted with 20% sucrose to obtain a series of six solutions possessing equal total weights of solute, in which the Ficoll concentration was continually increased at the expense of the sucrose concentration. After measuring the refractive indices and viscosities of these solutions, they were employed as solvents for measurements of the quantum yield and polarization of fluorescence of TNS (Figure 6). While the square of the refractive index of these solutions showed little or no variation with increasing amounts o Ficoll ($n^2 = 1.8583 \pm 0.0003$), the viscosities increased by 12-fold (1.655–19.53 cp) over the concentration range examined. The quantum yield of TNS fluorescence increased from 0.005 in 20% sucrose to 0.085 in 20% Ficoll, achieving a limiting value between a weight fraction of 0.2 and 0.4 of 20% Ficoll in 20% sucrose. The polarization of TNS fluorescence did not vary significantly in these mixtures. The deviation of the polarization value in 20% sucrose is attributable to experimental error resulting from the very low fluorescence intensity.

Solvents which influence the emission spectra of fluorescent solutes usually cause simultaneous shifts of λ_{max} to longer wavelengths and broadening of the emission band (Pringsheim, 1949). To see if TNS exhibited these changes, peak widths were plotted *vs.* the wavenumber of maximal emission (ω_{max}) for all the solvents which had been examined (Figure 7). Although a linear relationship in the predicted direction is found within the wavelength region of 410 and 470 mμ, at longer wavelengths the peak widths become smaller. The two experimental points which fall between the limbs of the curve were obtained in the only solvents which were examined without purification (*N*-methylformamide and glycerol). Despite the fact that reagent grade solvents were used in both cases, it is possible that the

TABLE III: Quantum Yields and Related Parameters of TNS Fluorescence in Aqueous Protein Solutions.[a]

Soln	Concn of Protein (mg/ml)	Buffer, pH	Quantum Yield (ϕ)	Emission Max λ_{max} (mμ)	Emission Max ω_{max} ($cm^{-1} \times 10^{-4}$)	Band Width[b] ($\Delta\omega$) (cm^{-1})
Bovine plasma albumin	10[c]	Tris, 8	0.34	446	2.22	5430
β-Lactoglobulin	10[c]	Acetate, 5	0.36	426	2.35	4110
β-Lactoglobulin	10[c]	Tris, 8	0.56	427	2.34	4330
Lysozyme	10	Acetate, 5	0.04	455	2.20	5800
γG-Immunoglobulin	9.2	Tris, 8	0.05	450	2.22	5430
Ovalbumin	10	Tris, 8	0.06	434	2.30	5180
Urease	2	Tris, 8	0.09	453	2.21	5650
α-Chymotrypsin	10[c]	Chloride, 2.3	0.18	446	2.24	5450
Chymotrypsinogen A	9.9[c]	Chloride, 2.3	0.07	436	2.29	5460
Trypsin	10[c]	Chloride, 2.3	0.18	447	2.24	5240
Trypsinogen	10[c]	Chloride, 2.3	0.18	445	2.25	5380
Pepsin	10	Acetate, 5	0.02	462	2.16	5660
Pepsinogen	9.4	Tris, 8	0.04	445	2.25	6240

[a] Emission excited at 366 mμ, 25°. [b] Peak area divided by the peak height. See text for details. [c] "Saturated." See text for details.

poor agreement of these two points is due to undetected solvent contamination.

Preliminary experiments in which TNS fluorescence was examined in aqueous solutions of several proteins indicated that proteins can cause marked increases in the observed quantum yield, verifying the observations of Weber and Laurence (1954). Protein solutions of *ca.* 10 mg/ml in suitable buffers were used as solvents in which to measure the peak width, λ_{max}, and quantum yield of TNS fluorescence (Table III). In order to maximize the binding of TNS to the protein in question, molar ratios of protein to TNS were maintained between a minimum of 12–16 and a maximum of *ca.* 70 except for urease (0.5) and γG-immunoglobulin (6.8). Most protein solutions were tested to determine the extent of TNS binding by dissolving an additional 10 mg of solid protein in the protein solution (volume, 4 ml) immediately after fluorescent measurements were completed. The fluorescent emission spectrum was then recorded and compared with the spectrum obtained with the original solution. If no increase in fluorescent intensity was noted, the solution was considered "saturated"; if an increase was noted, the solution was considered to be "not saturated." Protein solutions which were "saturated" under the conditions given in Table III were bovine plasma albumin, trypsin and trypsinogen, chymotrypsin and chymotrypsinogen, and β-lactoglobulin (at both pH 5 and 8). Solutions of ovalbumin, urease, pepsin and pepsinogen, lysozyme, and γG-immunoglobulin were "not saturated."

Different proteins enhanced TNS fluorescence to different extents. Under the conditions employed in these experiments, quantum yields >0.1 were obtained in solutions of bovine plasma albumin, trypsin, trypsino-

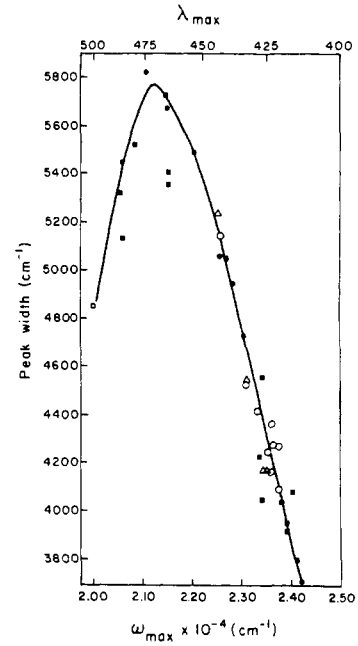

FIGURE 7: The correlation between effective peak width and the position of maximal emission for TNS fluorescence in organic solvents. Key: □, water; ○, alcohols; △, acids; ●, dioxane–water mixtures; and ■, miscellaneous. All data were collected at 25° using 366-mμ excitation. Proceeding from water at 500 mμ to the peak of the curve, the points represent water, 60% sucrose, formamide, 30% dioxane, phenol, 40% dioxane. The points on the descending limb of the curve can be identified by reference to Table II. λ_{max}, wavelength of maximal emission; ω_{max}, wavenumber of maximal emission.

gen, chymotrypsin, and β-lactoglobulin. The remaining proteins of Table III elicited quantum yields of <0.1. The variation of TNS emission spectra in these protein solutions was much less than that observed in the organic solvents; *e.g.*, whereas λ_{max} values in the solvents ranged from 413 to >480 mμ, λ_{max} values in the protein solutions covered a range from 426 to 460 mμ. A similar restriction can be noted in the range of band widths observed in the protein solutions.

Discussion

The work presented above indicates that TNS is practically nonfluorescent in water but fluoresces with different quantum yields in organic solvents or aqueous solutions of different native proteins. It is this property which makes TNS useful as a probe of protein conformation. Several lines of evidence favor the notion that hydrophobic interactions are responsible for enhancement of TNS fluorescence. They include: (1) the increases in quantum yield and decreases in bandwidth and wavelength of maximal emission with decreases in the dielectric constant of the solvent; (2) the increase in fluorescent intensity upon denaturation of proteins in the presence of TNS (Gally and Edelman, 1965; Alexander and Edelman, 1965); (3) the binding of the related compound ANS to the hydrophobic heme crevice of apomyoglobin (Stryer, 1965); (4) the high quantum yield found in solutions of proteins known to possess hydrophobic binding sites. This evidence will be discussed below in considering solvent effects and their rationalization in terms of the mechanism of TNS fluorescence.

In all of the homologous solvent series, increasing the number of methyl or methylene groups in the solvent structure resulted in higher quantum yields of TNS fluorescence. Concomitantly, both the wavelengths of maximal emission and the effective band widths were decreased. The portion of the solvent molecule responsible for the effects upon the position and shape of the emission band is probably the hydrocarbon chain itself, rather than any of the polar functional groupings. This is best exemplified by comparing the fluorescence data obtained from studies in the primary alcohols and acids (Table II). Changes in both λ_{max} and band width are correlated with the number of methyl or methylene groups, regardless of the nature of the functional groups in the two series of solvents; *e.g.*, the fluorescence of TNS dissolved in acetic acid is indistinguishable in peak position and width from the fluorescence observed in methanol; these parameters are also identical in propionic acid and ethyl alcohol, and in *n*-butyric acid and *n*-propyl alcohol.

The interpretation of changes of quantum yield with solvent structure appears to be less straightforward than the interpretation of changes in λ_{max} and the band width. Although a broad emission band at long wavelengths is never accompanied by a sizable quantum yield, the converse is by no means true: a narrow emission band at short wavelengths is frequently accompanied by low quantum yields. The relationships between quantum yield and λ_{max} found in the primary aliphatic alcohols seem to set an upper limit upon the quantum yield to be expected at a given λ_{max}, since none of the solutions which we have examined have exceeded the limits set by these alcohols; *e.g.*, the acids promote quantum yields which are lower than those observed in the alcohols. This suggests that the carboxyl group is quenching the observed fluorescence, a process known to occur in some fluorescent species (Weber and Wold, 1963). The data in Table II indicate that solvents such as the amines and pyridine are extremely effective quenching agents, despite the very low value of λ_{max} and the narrow peaks which are observed. Keto groups appear to be effective quenchers. Unsaturation also decreases the quantum yield (allyl alcohol, pyridine). In general, solvents possessing delocalized electrons are fairly effective as quenching agents.

These considerations suggest that even the highest quantum yields observed might be exceeded in pure aliphatic hydrocarbons, which contain no delocalized electrons. Unfortunately, the insolubility of TNS precluded measurements of quantum yields in such solvents.

In homologous solvents, an increase in the number of methyl or methylene groups in a solvent molecule should lower the polarizability and the dielectric constant. The correlation between fluorescence properties (λ_{max}) and the dielectric constant in the series of primary aliphatic alcohols is quite good. To examine this correlation further in a system which exhibited negligible changes in viscosity and density, the fluorescence properties of TNS in dioxane–water mixtures were studied. A correlation was observed between fluorescence properties and the dielectric constant. Only in the region of fairly high dioxane concentrations was any deviation noted between the dielectric constant and λ_{max}, and this region is known to be associated with several anomalies. Stallard and Amis (1952), who studied the thermodynamic properties of dioxane–water mixtures, reported very rapid changes in such properties as the heat of vaporization between 90 and 100% dioxane. Tommila and Koivisto (1948) found a maximal difference between the partial molal volume of dioxane–water mixtures and pure dioxane at 0.80 mole fraction dioxane (95 wt %). These effects are probably due to strong ordering of the structure of dioxane by small amounts of water, possibly mediated *via* dipole-induced dipole bonds (Stallard and Amis, 1952). If so, the effects on TNS fluorescence are explicable by a marked change in solvent structure at dioxane concentrations of >70%. It is also possible that the highly polar TNS molecule could form dipole-induced dipole bonds with nearby dioxane molecules and thereby contribute to a local difference in the solvent structure.

TNS fluorescence is affected by characteristics of the solvent other than the dielectric constant. In addition to the influence of specific chemical groupings (see above), the bulk viscosity of the solvent probably plays a role in enhancing the fluorescence of TNS. This view is supported by the data of Figure 2, which demonstrate a

dramatic difference in the fluorescence of solutions of NAS and TNS in response to changing values of T/η. In addition, the experiments carried out at constant temperature in sucrose–Ficoll mixtures of constant refractive index show that increases in quantum yield accompany increases in the viscosity of the solvent.

A mechanism of TNS fluorescence may be proposed in terms of the spectral characteristics and solvent effects discussed above. When TNS is excited in the absorbance band of longest wavelength the transition almost certainly involves the promotion of a π electron to an antibonding π orbital ($\pi \rightarrow \pi^*$). This assignment is supported by two lines of evidence. First, the large positive value of the polarization observed in glycerol suggests that the absorbance and emission oscillators of TNS are nearly parallel, a geometry known to exist in a $\pi \rightarrow \pi^*$ transition. Second, the blue shift observed with decreasing dielectric constant in homologous solvents is commonly observed with $\pi \rightarrow \pi^*$ transitions. The possibility of an $n \rightarrow \pi^*$ transition occurring in the 360-mμ absorbance band of TNS would seem very unlikely since solvents which are capable of hydrogen bonding caused shifts of λ_{max} to longer wavelengths, whereas in such solvents an $n \rightarrow \pi^*$ transition is usually characterized by shifts of λ_{max} toward shorter wavelengths.

The minor changes in the absorbance spectrum caused by two solvents of very different quantum yield (water and ethyl alcohol) would suggest that ground state interactions have only a small role in determining the width and maximum of the fluorescence of TNS. The major effect upon these properties probably results from solvation of the excited states of TNS. This hypothesis is consistent with the changes observed in various solvents. A theory due to Lippert (1957) can be used to calculate the difference in dipole moment between the ground state and the first singlet excited state of TNS from the effects of different solvents on λ_{max}. The dipole moment in the first singlet excited state was calculated from present data to be *ca.* 10 D. greater than that of the ground state. Since the excited state in TNS is more polar than the ground state, excited molecules would be expected to interact more strongly with polar solvents than would molecules in the ground state. In solvents of increasing polarizability and dielectric constant, this interaction would decrease the energy difference between the excited and ground states by lowering the energy of the first excited singlet. If the first excited singlet species is solvated, the Franck–Condon principle requires that the ground state molecule which is obtained immediately following emission must be equally solvated, since molecules in the solvent shell of TNS cannot appreciably alter their positions during the time required for fluorescence to take place. It would be expected that large decreases in the energy of the first excited singlet would be accompanied by a maximal number of interactions with the solvent, which would result in a very large number of vibrational quanta in the state resulting from emission. Since the number of vibrational levels in the state obtained upon emission will determine the shape of the emission spectrum, a good correlation should exist between decreasing energies of emission (increasing λ_{max}) and an increasingly broad fluorescence spectrum.

The variation of the quantum yield with changes in solvent can be explained by an hypothesis which invokes three mechanisms of nonradiative deactivation of the excited state. The first of these has already been implied above: if the first excited singlet interacts with the solvent, the probability of nonradiative transitions to the ground state will be increased. Solvents which exhibit minimal interaction with the excited molecule should allow the highest quantum yields, and one would predict the observed correlation between decreasing λ_{max} and increasing quantum yields in structurally related solvents.

A second means of nonradiative deactivation is afforded by intramolecular motion (Lewis and Calvin, 1939). Weber and Laurence (1954) have reported that several aminonaphthalenesulfonates which were fluorescent in both water and organic solvents could be deprived of their ability to fluoresce in water, but not in organic solvents, by conversion to the corresponding anilinonaphthalenesulfonates. Förster (1946) has suggested that fluorescence of the anilinonaphthalenesulfonates is observed only when the two rings are planar. Our results indicate that increasing solvent viscosity will increase the quantum yield of these compounds. This result supports the work of both Weber and Laurence (1954) and Oster and Nishijima (1956) and strengthens the hypothesis that intramolecular rotation between the phenyl and naphthyl rings of TNS is causing internal losses of energy.

A third path of deactivation is afforded by chemical quenching, which can be invoked to explain many of the observed differences from solvent to solvent. Chemical quenching by the carboxyl group of the fatty acids is probably responsible for the decreased quantum yields in these solvents when compared to the analogous alcohols. The very effective quenching by bases such as diethylamine suggests that proton transfers from the excited state may be occurring in these systems (Weller, 1958).

In addition to these three paths of radiationless deactivation, intersystem crossing to the triplet state also may occur. As yet, no information is available concerning this possibility.

Proteins appear to bind TNS to sites which are hydrophobic in nature. Although TNS is charged, the charged group does not appear to play a large role in binding. A positively charged derivative of ANS, *N*-(1-anilinonaphthalene-4-sulfonyl)-1,6-diaminohexane, has been observed to bind as well as ANS itself to heavy chains of human γG-immunoglobulin (Gally, 1964). In aqueous solutions of proteins, only bound TNS will contribute to the fluorescence. The results presented in Table III indicate that those proteins known to possess hydrophobic binding sites (bovine serum albumin, chymotrypsin) cause a marked increase in TNS fluorescence. Chymotrypsinogen, which is known to bind inhibitors and virtual substrates much less tightly than does chymotrypsin (Vaslow and Doherty, 1953;

Deranleau, 1966) causes a much lower quantum yield than that observed with the active enzyme.

The high quantum yields observed in neutral and acidic solutions of β-lactoglobulin extend the observations of Klotz (1949) that this protein possesses an affinity for organic anions. The emission spectrum of TNS in β-lactoglobulin solutions in pH 8 Tris is characterized by an emission maximum, effective peak width, and quantum yield which are very similar to those observed for TNS fluorescence in *n*-propyl or *n*-butyl alcohol. The resemblance of the spectral characteristics raises the possibility that TNS is being bound to a site in β-lactoglobulin which resembles an aliphatic alcohol. The quantum yield observed in β-lactoglobulin solutions decreases when the pH is decreased from 8 to 5. Since the peak width and λ_{max} do not vary with this change in conditions, it is possible that a group in the binding site has altered its ionization state, with the formation of a quenching group where none existed previously. Alternatively, the conformation of the site may be slightly modified due to changes in the ionization of groups spatially removed from the site with either a concomitant introduction of quenching groups into the vicinity of the TNS or a decrease in the extent of TNS binding.

Changes in protein conformation can be readily followed by measuring TNS fluorescence. Some recent applications include investigations of the thermal transitions of Bence–Jones proteins (Gally and Edelman, 1965), of the acid expansion of bovine serum albumin (Weber and Young, 1964), and of the changes in conformation observed upon activation of pepsinogen and chymotrypsinogen (Alexander and Edelman, 1965).

At the present time very few methods are available with which to study hydrophobic residues and their contribution to the conformation of protein molecules. It is hoped that a series of fluorescent probes related to TNS can be developed for the study of specific interactions of hydrophobic residues.

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Inhibition of Collagen Intermolecular Cross-Linking by Thiosemicarbazide*

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ABSTRACT: Thiosemicarbazide (TSC) reacts at acid pH with embryonic calf skin collagen to yield a product which contains 2 moles of thiosemicarbazide/mole of collagen. This collagen derivative polymerizes normally from solution at neutral pH but the fibrils readily dissolve in the cold, similar to those of lathyritic collagen. After gel filtration or CM-cellulose chromatography denatured thiosemicarbazide collagen shows little loss of TSC, the latter being distributed among the α and β components. Comparing the TSC–collagen and normal collagen no decrease in cross-linked components is observed. Difference spectra suggest that the TSC is bound to the collagen as thiosemicarbazone derivatives.

The 2 moles of TSC are found on the "A" fragment obtained after removal of 25% of the molecule at the "B" end with tadpole collagenase. Digestion of the native protein with trypsin or chymotrypsin removes most of the TSC, which is present in two different groups of fragments of several thousand molecular weight. Lathyritic chick embryo collagen produced *in vivo* by thiosemicarbazide contains negligible amounts of this lathyrogen. A significant feature of these studies is that TSC–collagen behaves like lathyritic collagen with respect to impaired intermolecular cross-linking but has no deficiency of intramolecular cross-links suggesting that the two properties are not necessarily interdependent.

The age and the solubility characteristics of various collagen fractions in tissue have often been related to their degree of inter- and intramolecular cross-linking (see review by Harding, 1965). The extractable collagen[1] from lathyritic animals is deficient in intramolecular cross-links (Martin *et al.*, 1961), and forms unstable native fibrils which dissolve in cold salt solutions (Gross, 1963). This latter characteristic indicates an impairment of intermolecular cross-linking and probably underlies the marked loss of tissue tensile strength and increased collagen solubility in lathyritic animals (Levene and Gross, 1959). All other physical and chemical features of lathyritic collagen appear normal, implying the presence of a subtle molecular alteration.

Recently, attention has focused upon the presence of aldehyde-like compounds in collagen (Rojkind *et al.*, 1964) and their potential role in cross-linking. All of the lathyrogens are capable of forming addition products with aldehydes (Levene, 1962) but it has been clearly demonstrated that lathyritic collagen does not contain significant amounts of bound lathyrogen (Orloff and Gross, 1963).

Recent work suggests that one type of intramolecular cross-link may develop simultaneously with the conversion of specific lysines in collagen to aldehyde derivatives (Bornstein *et al.*, 1966). In addition, lathyritic collagen was found to lack these carbonyl compounds but to possess the specific precursor lysyl residues.

In our studies of the binding of the lathyrogen, thiosemicarbazide, to collagen we noted a marked inhibition of intermolecular cross-linking of fibrils formed from TSC–collagen similar to that seen for lathyritic collagen fibrils. This report describes these phenomena and discusses the implications.

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[1] Abbreviations are TSC, thiosemicarbazide; TCA, trichloracetic acid; TC^A, tropocollagen "A" fragment; TC^B, tropocollagen "B" fragment; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.