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Neutral and Cationic Sulfonamido Derivatives of the Fluorescent Probe 2-*p*-Toluidinylnaphthalene-6-sulfonate. Properties and Mechanistic Implications†

Frank C. Greene

ABSTRACT: The sensitivity of the fluorescence energy of 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) to apparent solvent polarity is considerably greater in methanol–water mixtures and lower primary alcohols than in higher primary alcohols or solvents containing no hydroxyl groups. It is suggested that the behavior of TNS in lower alcohols and their mixtures with water is an anomalous result of the combination of general polarity influences and *changes* in the nature of specific interactions of the probe with hydroxyl groups of such solvents. Upon conversion of the sulfonate group of TNS to sulfonamido, the biphasic behavior observed in alcohols is eliminated, and fluorescent probes are obtained which have more nearly equal sensitivities to polarity in the alcohols and nonhydroxyl solvents. The sulfonamido fluorescent probes are more sensitive to general environmental polarity than TNS, and are superior as probes of environmental polarity.

Fluorescent probes (environmentally sensitive fluorophores) of the *N*-arylaminonaphthalenesulfonate type are widely utilized in contemporary biochemical research, and have proven quite useful, despite some uncertainty concerning the nature of the mechanisms determining their sensitivity (Brand and Gohlke, 1972). Interpretations of the fluorescence characteristics of these probes in pure solvents are based on the premise that the energy and efficiency of emission reflect the degree of unrestrained solvent–excited probe interaction (McClure and Edelman, 1966), and may be taken as indications of microenvironmental polarity (Turner and Brand, 1968). Such interpretations are often extended to studies involving the binding of probes by macromolecules, though additional factors of environmental restraint may be operative (Ainsworth and Flanagan, 1969). In the present investigation, neutral and cationic derivatives of the probe 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS)[1] have been prepared as aids in the study of protein–amphi-

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[1] Abbreviations and symbols used are: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; II, 2-*p*-toluidinylnaphthalene-6-sulfonamide; III, 2-*p*-toluidinylnaphthalene-6-[*N*-β-ethylamine hydrochloride]sulfonamide; 1,8-ANS, 1-anilinonaphthalene-8-sulfonate; 2,6-ANS, 2-anilinonaphthalene-6-sulfonate.

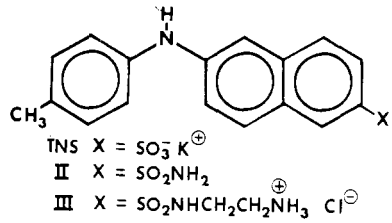


FIGURE 1: Fluorescent probe formulas.

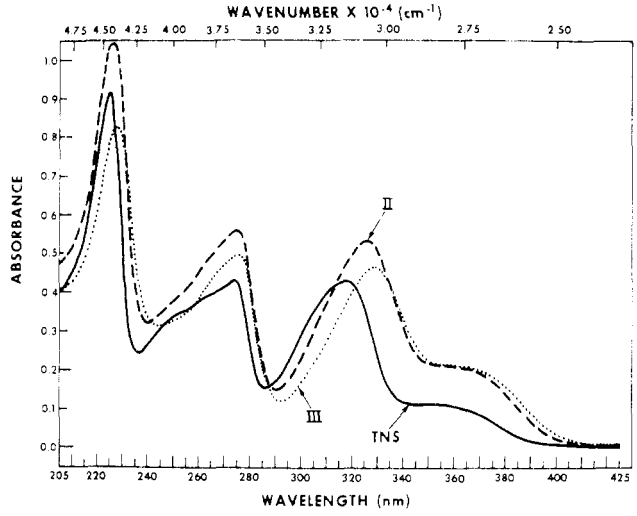


FIGURE 2: Ultraviolet absorption spectra of TNS, II, and III in 1-propanol; concentration, 2×10^{-5} M.

phile interactions. These derivatives possess fluorescent probe characteristics, but their polarity sensitivities differ markedly from those of the parent TNS. This paper describes some of the characteristics of the sulfonamido TNS fluorescent probes, and explores mechanistic implications of their differences from TNS.

Results

Synthesis. In a modification of the procedure of Cory *et al.* (1968), TNS was activated *via* its sulfonyl chloride and coupled to ammonia and ethylenediamine, respectively, to yield sulfonamido (II) and *N*-(β-aminoethyl)sulfonamido (III) derivatives (Figure 1). Details are contained in the Experimental Section.

Absorbance Properties. Ultraviolet absorption spectra of TNS and the sulfonamido derivatives II and III are presented in Figure 2. The spectra of II and III have less vibrational fine structure in the 250–275-nm region, show increased intensity in their lowest energy bands, and are generally red-shifted compared to the spectrum of TNS. The largest shift occurs in the second lowest energy band. This shifting with modification of the sulfonate at the 6 position of the naphthalene ring suggests that the corresponding electronic transition is longitudinally polarized, and a ^1L_b assignment is assumed, after Hirshberg and Jones (1949) and Jaffé and Orchin (1962). Seliskar and Brand (1971a), however, suggest that the two lowest energy absorption bands exhibit charge-transfer characteristics, with a transition of the $1 \rightarrow a_\pi$ type. The two highest energy bands are probably ^1B_b and ^1L_a, respectively, after Klevens and Platt (1949). The observed spectral changes are consistent with a greater capacity of the sulfonamide group than sulfonate for resonance interaction with the aromatic ring system.

Figure 3 shows the position (ν_{max}) of the major absorption bands of the three probes in methanol, 1-propanol, and 1-dodecanol, along with the positions of the TNS and III bands in aqueous solution. With regard to solvent sensitivity, the general effect on the spectra of these probes in alcohol solvents (Figure 3) is a red-shifting proportional to decreasing static dielectric constant, and the sulfonamide probes are somewhat more solvent sensitive than TNS. Spectra recorded in dioxane, ethyl acetate, and acetonitrile were generally similar in structure to those recorded in alcohols.

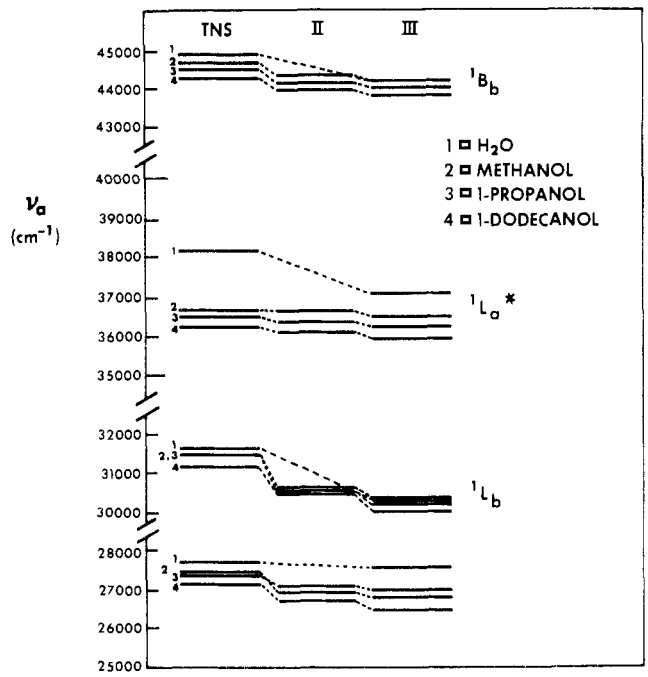


FIGURE 3: Positions of major absorption bands of TNS, II, and III in different solvents. The asterisk indicates the shape of the ^1L_a envelope is significantly different in water than in alcohols.

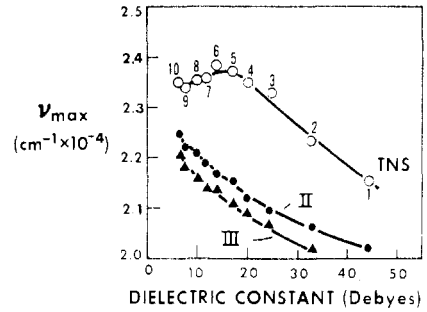


FIGURE 4: Relation of fluorescence energy of probes to static dielectric constant in primary alcohols and a 3:1 methanol–water mixture: (○) TNS, 1.33×10^{-6} M; (●) II, 8.7×10^{-7} M; (▲) III, 7.3×10^{-7} M. Table I gives the solvent numbering index.

Fluorescence Properties. The relations of fluorescence energy parameters of the probes to solvent polarity in a solvent series composed of primary alcohols and a methanol–water mixture are compared in Figures 4 and 5, for macroscopic scales based on static dielectric constant (Maryott and Smith, 1951; Akerlöf, 1932) and on the orientation polarization term described by Lippert (1957). Table I gives the solvent numbering index. The dielectric constant term in both these polarity parameters requires, for proper interpretation of the fluorescence data, that the solvent dipoles be capable of relaxing to equilibrium distributions during the lifetime of the solute excited state. In order to evaluate

Table I: Solvent Numbering Index.

No.	Solvent	No.	Solvent
1	Water–methanol (3:1, v/v)	13	*N*,*N*-Dimethylformamide
2	Methanol	14	Acetic anhydride
3	Ethanol	15	Propionic anhydride
4	1-Propanol	16	Cyclohexanone
5	1-Butanol	17	Tetrahydrofuran
6	1-Pentanol	18	Pyridine
7	1-Heptanol	19	Ethyl acetate
8	1-Octanol	20	*p*-Dioxane
9	1-Decanol	21	Diethyl ether
10	1-Dodecanol	22	Benzene
11	Acetonitrile	23	Cyclohexane
12	Acetone	24	*n*-Hexane

this possibility in the alcohols, natural fluorescence lifetimes, τ_0, were calculated (Strickler and Berg, 1962) for TNS, II, and III, and compared to dielectric relaxation times measured for primary alcohols by Garg and Smyth (1965). The fluorescent lifetime values were clustered in the 20–25-nsec range. A calculation of the fluorescent lifetime of II by the method of Berlman and Walter (1962) indicated a value of 17 nsec. The dielectric relaxation times, attributed to breaking of hydrogen bonds in molecular aggregates followed by ROH rotation, range from 0.1 nsec for 1-propanol, through 2.0 nsec for 1-decanol, all at 20°. The solvent relaxation times are at least an order of magnitude smaller than the probe fluorescence lifetimes. In addition, the emission energy (ν_{max}) of TNS in 1-decanol does not change between 25 and 45°, though the solvent dielectric relaxation time increases by a factor of three. It thus appears that the solvent relaxation condition is satisfied, and that meaningful interpretations of the fluorescence behaviors are possible.

In each case of Figures 4 and 5, the polarity sensitivity of a probe is taken as proportional to the absolute slope of the appropriate solvent polarity *vs.* solute fluorescence energy curve. From these figures, the TNS polarity sensitivity is greatest in the high polarity region of the scale, whereas in the middle and low polarity regions, its sensitivity goes to zero, and the data become equivocal. The sensitivities of probes II and III, though less than that of TNS in the high polarity region, are more linearly related to solvent polarity, however, and are greater than that of TNS in the middle and low polarity regions of the scale. It is also apparent, from a comparison of Figures 3 and 5, that the observed polarity sensitivities result from excited state processes.

In the relation:[2]

$$\nu_a - \nu_f = \frac{2\left[\frac{\epsilon - 1}{2\epsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right]}{hc_0A^3}(\mu_e - \mu_g)^2 + \text{constant} + \text{smaller terms}$$

derived by Lippert (1957) and Mataga *et al.* (1956), the nonradiative loss term ($\nu_a - \nu_f$) is proportional to the tendency of the solvent to interact with, and influence the energy of, the excited state during the interval between absorption and emission. The relation can also be used to estimate dipole moment changes ($\mu_e - \mu_g$) accompanying the emission process. Accordingly, an apparent dipole moment change of ~44 D has been estimated for TNS behavior in the high polarity region of the alcohol scale, in agreement with results of Seliskar and Brand (1971b), and an apparent value of zero for TNS in the low polarity region. The more nearly linear polarity sensitivities of II and III indicate $\mu_e - \mu_g$ changes of ~15 D. The sensitivities of fluorescence quantum yields (ϕ_f) of the respective probes to solvent polarity, also displayed in Figure 5, vary in the same way as those of fluorescence energy, suggesting that these processes are mechanistically linked in the alcohols.

In nonhydroxyl-containing solvents (Figure 6), the fluorescence behavior of the sulfonamido probes, as exemplified by II, is similar to that observed in alcohols; the polarity sensitivity is similar, and a $\mu_e - \mu_g$ value of ~16 D is obtained. TNS behavior in this nonhydroxyl series is different

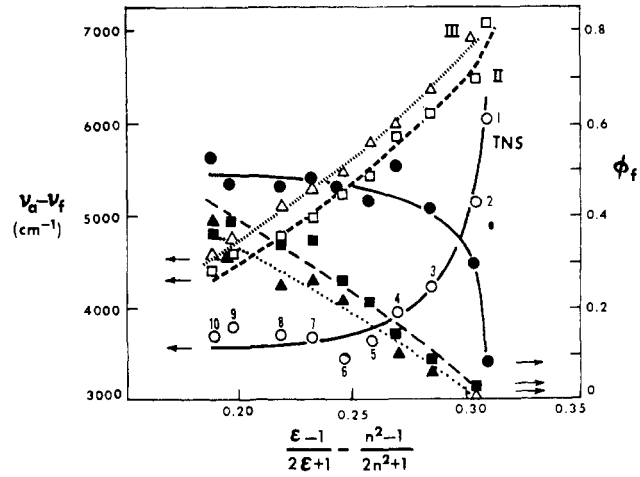


FIGURE 5: Relation of energy loss factor ($\nu_a - \nu_f$) and relative quantum yields (ϕ_f) of probes to solvent orientation polarization in alcohols and a 3:1 methanol–water mixture: (○, ●) TNS; (□, ■) II; (△, ▲) III. Probe concentrations same as in Figure 4.

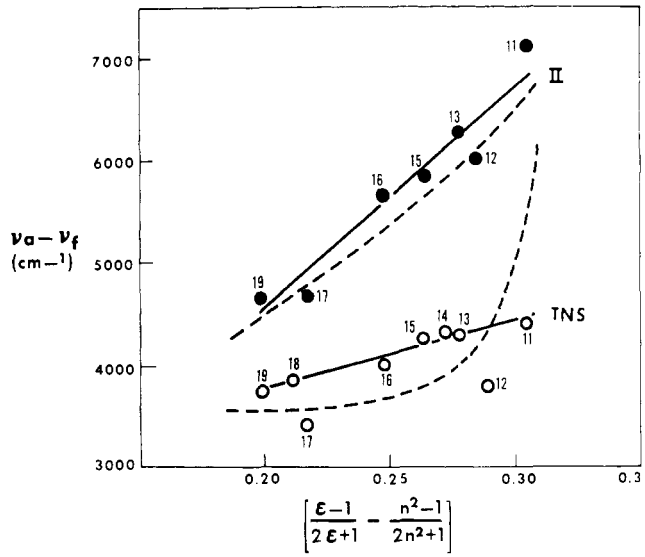


FIGURE 6: Relation of energy loss factor ($\nu_a - \nu_f$) of probes to solvent orientation polarization in nonhydroxyl solvents: (○) TNS; (●) II. Probe concentrations 5 × 10^{-6} M. Dashed lines indicate equivalent relations in the alcohol series.

[2] Where: ν_a and ν_f = absorption and fluorescence wave numbers, respectively; ϵ = static dielectric constant; n = refractive index; μ_e and μ_g = excited and ground state dipole moments, respectively; h = Planck's constant; C_0 = speed of light *in vacuo;* and A = radius of the solute Onsager cavity (assumed equal to radius of sphere equivalent to solute molecular volume).

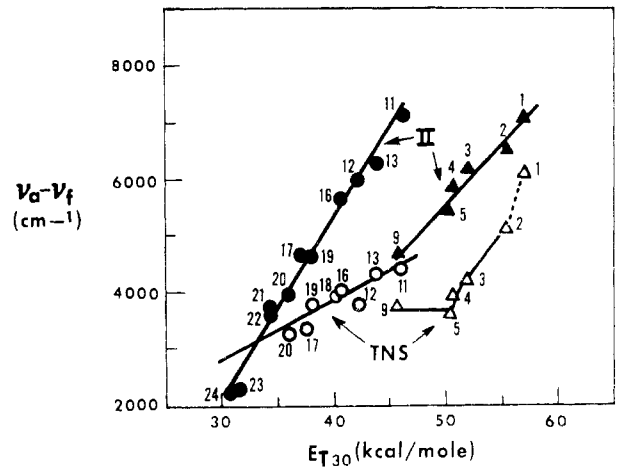


FIGURE 7: Relation of energy loss factor ($\nu_a - \nu_f$) of probes to empirical solvent polarity parameter $E_{t,30}$ in 3:1 methanol–water mixture and primary alcohols: (△) TNS; (▲) II; and nonhydroxyl solvents: (○) TNS; (●) II. Probe concentrations in alcohols same as in Figure 4, in nonhydroxyl solvents same as in Figure 6. The $E_{t,30}$ value for 1-decanol was estimated by matching its reported Z value (Foster, 1971b) to the Z *vs.* $E_{t,30}$ alcohol regression line (Reichardt and Dimroth, 1968).

from that observed in alcohols, however, in that the polarity sensitivity response is apparently linear with a $\mu_e - \mu_g$ value of ~9 D. This value, lower than that of the sulfonamido probes, and intermediate between the two extremes manifested by TNS in alcohols, is similar to the value of ~10 reported by McClure and Edelman (1966) from measurements in unspecified solvents. Measurements of the energy, bandwidth, and relative quantum yield of TNS fluorescence in 1-decanol indicated no change within the concentration range 5×10^{-8}–5×10^{-6} M, so excimer formation is not likely a cause of the apparent TNS sensitivity shift.

The fluorescence characteristics of TNS and II have also been evaluated with solvent polarities expressed on a microscopic scale $E_{t,30}$ (Reichardt and Dimroth, 1968), as suggested by Kosower (1958); see Figure 7. In this type of empirical polarity scale, polarity values are assigned to solvents on the basis of their effects on the charge-transfer absorption energy of a standard solute, and, ideally, provide a direct assessment of the polarity of the solvent cavity surrounding the solute. This scale is not linearly related to that used by Lippert, and, in addition, assigns generally higher polarity values to alcohols relative to nonhydroxyl solvents.[3] In spite of these differences, however, the conclusions drawn from representation on Lippert's orientation polarization scale can be sustained in the empirical one. A greater apparent polarity sensitivity for TNS than for II in the lower alcohols and methanol–water mixture (right of figure) is indicated by the greater slope of its curve in this region. A loss of TNS sensitivity in the higher alcohols is indicated by the similarity of the $\nu_a - \nu_f$ loss value for 1-butanol to that of 1-decanol (see also Figure 5). Retention of sensitivity by II in higher alcohols, however, is apparent from the energy interval of ~900 cm^{-1} between the loss values for 1-butanol and 1-decanol. In addition, II clearly shows greater polarity sensitivity than does TNS in the nonhydroxyl solvents (Figure 7, left).

In a preliminary experiment to test the possibility of deuterium isotope effects, the fluorescence of II was compared in CH_3OH and CH_3OD. The fluorescence yield of II in CH_3OD was 1.88 times that in CH_3OH. No difference in fluorescence energy was observed.

Discussion

It is apparent, from the experimental observations of this study, that sulfonamide derivatives of TNS exhibit higher polarity sensitivities than the parent probe in nonhydroxyl solvents and in higher primary alcohols. The apparent high sensitivity characteristic which makes TNS so popular as a fluorescent probe seems confined to a fairly narrow polarity range in a specific type of solvent (alcohol), and may thus constitute a special case.

It will be useful, in considering the behavior of TNS and its sulfonamido derivatives, to delineate general and specific solvent effects. For purposes of this discussion general effects may be considered as those which result from dipole–dipole and dipole–induced dipole interactions, and which can be predicted from the theoretical treatments of Lippert (1957) and Mataga *et al.* (1956). Other effects, *e.g.*, hydrogen bonding, that may result from the interaction of unique combinations of solute and/or solvent functional groups are specific effects. There will, in fact, be some probability of specific effects in the interaction of any two polar compounds. In the present study, the possibility of development of a net trend of such specific effects is minimized in the group of nonhydroxyl solvents. This occurs because the members of this group share a low tendency toward self-association, and represent a variety of different polar functional groups. In the polarity series composed of these solvents, therefore, influences of probe behavior are considered to be determined primarily by *general* polarity effects. The probe behavior in this series may be reasonably explained by the Lippert–Mataga theories, and the higher sensitivity of the sulfonamido probe II relative to TNS in nonhydroxyl solvents may be viewed as a consequence of a greater difference ($\mu_e - \mu_g$) in the dipole moments of the excited and ground states. From the similarity of its behavior to that of II in alcohols, it appears that III shares this characteristic.

The sulfonamido probes differ further from TNS in that, based on observations of II, their apparent polarity sensitivities are similar in the primary alcohol and nonhydroxyl solvent series. This behavior, and the near coincidence of the Lippert plot curves for II in the two solvent series (Figure 6), suggests that the sulfonamido probe–alcohol interactions *vary* in a manner dependent on general polarity characteristics. This behavior may also be viewed as a function of the concentration of polar solvent hydroxyl groups dispersed in a hydrocarbon matrix (see below). The behavior of TNS in alcohols, however, with its apparent transition

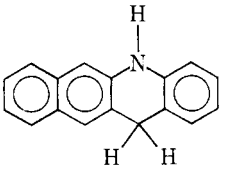
[3] The displacement, on the empirical $E_{t,30}$ scale (Figure 7), of the polarity sensitivity curves for TNS and II in alcohol solvents from those in nonhydroxyl solvents is an indication that differences in the nature of specific solvent–solute interactions exist between the arylaminonaphthalenesulfonate probes and the standard solute (2,4,6-triphenyl-*N*-[3,5-diphenyl-4-hydroxyphenyl]pyridinium betaine) used to define the $E_{t,30}$ scale. Kosower and Tanizawa (1972) reported a similar finding for 9,9′-dianthryl; similar displacements are also apparent when the data of Turner and Brand (1968) for 1,7-ANS and Chen (1967) for dansyltryptophan are displayed on the $E_{t,30}$ scale. The alcohol and nonalcohol regression lines also differ significantly in a comparison of the $E_{t,30}$ and Z scales (Reichardt and Dimroth, 1968). It is likely that the differences between these compounds and the $E_{t,30}$ standard solute result from differences in modes of solute interaction with alcohols rather than in interactions with nonhydroxyl solvents. While placing limitations on the generality of the empirical polarity scale, they also clearly indicate the significance of specific microenvironmental effects. The hypothesis developed in this study suggests that such effects are operative in the observed differences in behavior between TNS and its sulfonamido derivatives.

from a high sensitivity interaction at high polarity values to a low or zero sensitivity interaction at low polarity values,[4] is anomalous, and indicates the involvement of factors other than general polarity effects.

It is probable that the behavior of TNS in alcohols is a specific process involving change in its mode of interaction with solvent hydroxyls. The sulfonamido probes apparently do not experience such a change. A reasonable model for these behaviors may be developed from the proposal that excited state species of both types of probes can participate in specific interactions with hydroxyl groups of alcohol solvents, but that these interactions diminish or vanish at low polarity values (low hydroxyl concentrations) in the case of TNS. In this way, the apparently higher polarity sensitivity observed for TNS in lower alcohols and alcohol–water mixtures can be interpreted as the sum of a low general polarity sensitivity, and relief from quenching and energy effects as the probe shifts to a noninteracting state.

The probability of specific interactions between polar solutes and hydroxyl-containing solvents has been reported in several publications (McRae, 1958; Walker *et al.*, 1967; Mataga *et al.*, 1956; Foster, 1971a). Regarding fluorescent probes, Camerman and Jensen (1970) have suggested that hydration may be important in the behavior of TNS, and that H · · · N interaction in strongly hydrogen bonding solvents could be involved. Brand *et al.* (1971) have noted the possibility of specific effects in the fluorescence of *N*-phenyl-2-aminonaphthalene in ethanol–cyclohexane mixtures. In the present study, some indication of specific complexing between sulfonamido probes and hydroxyls is given by the preliminary observation (see above) that the quantum yield of fluorescence of II is higher in CH_3OD than in CH_3OH. The alcohol series (C_1–C_{12}) employed in the present study can be considered to represent variation of hydroxyl concentration in a hydrocarbon matrix, since an approximately linear relation exists between their static dielectric constants and hydroxyl concentrations. If, then, the apparent dependence of probe fluorescence properties on solvent hydroxyl concentration is regarded as resulting from specific quenching processes, the behaviors of II and III indicate specific interactions over the whole [OH] range studied. TNS may participate in such interactions at high [OH], but apparently does so only minimally at low [OH]. Walker *et al.* (1967) have studied the fluorescence behavior of indole and substituted indoles in 1-butanol–pentane mixtures, and have reported evidence for the formation of butanol–indole exciplexes. The fluorescence of these exciplexes is red-shifted with respect to uncomplexed indoles, and is subject to further red-shifting with increasing alcohol concentration in the solvent mixtures. The present study, in alcohols, is experimentally analogous to theirs, and similar interpretations may apply. Thus, polarity-sensitive emission of all probes may occur from specifically complexed states, and polarity-insensitive fluorescence of TNS may occur as a result of a shift to a noncomplexed state.

Such TNS states may be equivalent to the high and low sensitivity conformations, respectively, proposed in the scheme of Kosower and Tanizawa (1972). These authors suggested, on the basis of observations in dioxane–water mixtures, that the Franck–Condon excited state of TNS may relax to different emitting conformations depending on the polarity of the solvent.[5] These proposed conformations differ in the relative orientation of the two aromatic ring systems, and have different polarity sensitivities. It is possible that a high sensitivity conformation of TNS is stabilized through interactions with hydroxyl groups. Where the proposal of Kosower and Tanizawa implies a general mechanism, however, the present model is based only on specific interactions. An alternate possibility is that multiple emissions are being observed, and that the shifts in fluorescence maxima result, in part, from changes in the relative intensities of different emitting states. This possibility has been discussed for 1,8-ANS[1] by Spencer *et al.* (1969) and by Penzer (1972). Mataga *et al.* (1964) have observed that the fluorescence of indole, a *planar* molecule, is more polarity sensitive between ethanol and water than in a nonhydroxyl solvent series. These authors have proposed a mechanism of solvent-induced reversal of ^1L_a and ^1L_b states as an explanation. The possible involvement of intramolecular twist in the mechanism of TNS behavior might be resolved by a study of the fluorescent properties of 5,12-dihydro-5-azanaphthacene:



or a similar compound, which should be more hindered on its movement than TNS.

Implicit in this discussion is the suggestion that these special factors responsible for the behavior of TNS are less influential in the behavior of sulfonamido probes. How these differences derive from the modification of SO_3^- to SO_2-NHR is not certain, but it could involve additional stabilization of a charge-transfer state, or it might be due to relief of solvent structuring effects imposed by the SO_3^- group. In this latter regard, it is interesting that the apparent dipole moment change ($\mu_e - \mu_g$) of *N*-phenyl-2-aminonaphthalene in ethanol–water mixtures is 20 D compared to 40 D for 2,6-ANS[1] (Seliskar and Brand, 1971b).

With regard to the use of fluorescent probes in the study of macromolecules and membranes, TNS appears to be an "all or none" probe, capable of signaling the presence or absence of a given array of hydroxyl groups in its immediate vicinity. This is in some degree polarity sensitivity, but aside from this special response, TNS is relatively limited in its ability to indicate true environmental polarity. The TNS–sulfonamido probes, by virtue of higher general sensitivity and relative freedom from anomaly, appear to be superior to TNS as probes of binding site polarity.

Experimental Section

Synthesis of Sulfonamido Probes. TNS–chloride was prepared by a modification of the method of Cory *et al.* (1968), and allowed to react, without isolation, with an excess of either ethylenediamine or ammonium hydroxide to yield 2-*p*-toluidinylnaphthalene-6-[*N*-β-ethylamine hydrochloride]sulfonamide (III), or2-*p*-toluidinylnaphthalene-6-sulfonamide (II), respectively. The following preparation of III is an example: TNS (1.002 g, 0.00285 mol) was suspended in 20 ml of pyridine at room temperature. To this

[4] A leveling off of TNS fluorescence sensitivity with increasing alcohol size and a relative insensitivity to polarity change in nonhydroxyl solvents are apparent in the data of McClure and Edelman (1966).

[5] A similar proposal has been advanced by Penzer (1972) for 1,8-ANS.

mixture was added $POCl_3$ (0.75 ml, 0.00825 mol) dropwise with stirring. As the $POCl_3$ was added, the TNS dissolved, yielding a yellow-orange solution, and the flask became slightly warm to the touch. Stirring was continued for 50 min. The yellow-orange TNS–chloride solution was then added dropwise (over ~15 min) to 40 ml (0.6 mol) of ethylenediamine, yielding a slightly cloudy, yellow solution. Stirring was continued for 2 hr, and the yellow solution was poured into 450 ml of cold water, producing a fine, cream colored suspension. The suspension was centrifuged for 20 min at 13,000*g*, yielding a gel-like, white, fluorescing residue and a yellow mother liquor. The residue was washed three times with cold, dilute (pH 10) NaOH, and recovered from the third wash by centrifugation at 34,000*g*. This residue was suspended in 10 ml of 0.1 M HCl, and the pH adjusted to 4.0 with 1.0 M HCl. After addition of water to a final volume of 75 ml, the suspension was heated to 80° (dissolving most of the gel), and centrifuged at 31,000*g* in the preheated (65°) rotor of a Sorvall RC-2[6] centrifuge. The clear yellow centrifugate started to develop tiny platelets as it was being decanted out of the centrifuge tubes. A mixture of needle clusters and a silky, birefringent "milk" formed on cooling. The suspension was reheated until only needles and clear liquid remained and then stored in an insulated container. Crystallization proceeded and appeared complete after 3 days. The tiny needles were recovered by suction filtration using S+S no. 576 filter paper. The light yellow crystals were redissolved in water, and the crystallization and recovery procedure repeated. The second batch of crystals, white in color, was dried *in vacuo* at room temperature for 4 hr. The yield was 631 mg, or 57%.

Anal. Calcd for $C_{19}H_{22}N_3O_2SCl$: C, 58.23; H, 5.66; N, 10.72; O, 8.16; S, 8.18; Cl, 9.05. Found: C, 58.0; H, 5.77; N, 10.5; S, 8.29; Cl, 8.47.

The ir spectrum of III, by comparison to that of TNS, contained an additional broad band at 3000 cm^{-1}, which was assigned to the amine hydrochloride substituent, and broad sulfonamide bands at 1150 and 1315 cm^{-1}, replacing the 1180- and 1220-cm^{-1} sulfonate bands. Mass spectral analysis of the range 0–800 mass units indicated a mass of 355, equivalent to the free amine, and consistent with a total mass number of 391 for the hydrochloride. The total abundance of peaks above mass 358 was 3.2% of the 355 peak.

For preparation of II, 16 ml of concentrated NH_4OH (0.118 mol) was substituted for ethylenediamine in the reaction with TNS–chloride. The resulting suspension was recrystallized from acetone–H_2O as narrow, pale yellow platelets melting, without decomposition, from 219.5 to 221° (uncorrected). Mass spectral analysis (0–800 mass units) indicated a mass of 312. The total abundance of peaks above mass 315 was 0.23% of the 312 peak. Per cent yield was not calculated.

Anal. Calcd for $C_{17}H_{16}N_2O_2S$ (formula wt 312.39): C, 65.2; H, 5.23; N, 8.82; O, 10.35; S, 10.4. Found: C, 65.3; H, 5.16; N, 8.97; S, 10.25.

Thin-layer chromatography of TNS, II, and III on silica gel (Anisil S) revealed single fluorescent spots in each of two solvent systems. R_F values in 1-propanol–15 M NH_4OH (100:1) and 1-butanol–acetic acid–water (50:12:50, upper phase), respectively, were: TNS, 0.54 and 0.75; II, 0.94 and 0.95; III, 0.41 and 0.73 with slight tailing. That polymerization reactions involving coupling of TNS–sulfonyl chloride to the >N–H of a second TNS species would not occur to a significant extent in these syntheses is indicated by the low basicity of the nitrogen (the pK_b of diphenylamine is 13.12, for example), and by the reported (Cory *et al.*, 1968) inability of $(CH_3)_2SO_4$ or CH_3I to methylate the nitrogen of TNS. The elemental composition, mass spectra, and thin-layer chromatographic behavior of the sulfonamides isolated in the present study reveal no evidence of dimeric components.

Solvents used in this study were analytical or reagent grade, freed of fluorescent impurities by incubation with activated charcoal.

Absorption spectra were measured in a Cary 15 recording spectrophotometer equipped with a 1P28 phototube.

Fluorescence spectra were determined in a Turner 210 spectrophotofluorometer with sample compartment maintained at 25 ± 0.5°, unless otherwise noted. The excitation wavelength (λ_{ex}) was 350 nm. Relative quantum yields of fluorescence (ϕ) were determined by comparison to that of 2× recrystallized quinine sulfate according to the equation:

$$\phi_u = \phi_s \frac{\epsilon_s C_s \lambda_{ex,s} A_u}{\epsilon_u C_u \lambda_{ex,u} A_s}$$

(Turner, 1964), where s = standard, u = unknown sample, ϵ = extinction coefficient, C = concentration, and A = integrated fluorescence intensity. The quantum yield of quinine sulfate was taken as 0.57 at 348-nm excitation (Turner, 1966). Fluorescence lifetimes were calculated according to the method of Strickler and Berg (1962). In obtaining $\int \epsilon d\nu$, the integrated absorption intensity, it was assumed that the low-energy trailing edge of the probe absorption spectra contained only components of the lowest energy absorption band. The inflection point of this lower limb was visually located, and a tangent to the curve drawn at this point. An isosceles triangle was constructed, with the inflection point taken as the midpoint of one of the equal sides. The position of the apex of this triangle was taken as λ_{max}, and its area as $\int \epsilon d\nu$. An approximation of $\int \epsilon d \ln \nu$ was obtained as $1/\lambda_{max} \int \epsilon d\nu$. The accuracy of this method, estimated from trials with machine drawn gaussian curves (Cal Comp Plotter), was ±200 cm^{-1} for peak position, and ±10% for values of $\int \epsilon d\nu$.

Ionization characteristics of III and II were studied by automatic pH titration (Radiometer TTT 1C) and solubility properties. When 0.025 M KOH–0.08 M KCl was used to titrate a 3.3×10^{-4} M solution of III in 0.1 M KCl, one dissociable group was detected between pH 3.1 and 10. The titration of this group resulted in precipitation of the probe, and occurred in a very narrow pH region: the fractional dissociation changed from 0.091 to 0.91 in 0.68 pH unit, compared to 2.0 pH unit for a normal curve. The observed inflection point of pH 7.8 is thus not a true pK_a, but reflects solubility perturbations. Hysteresis effects could be demonstrated in the dissociation process. A solution of III prepared at pH 3, and titrated with minimal stirring, remained clear and nonfluorescent through pH 8.3, becoming cloudy and fluorescing blue by pH 8.9. In the back titration, however, a cloudy, fluorescent suspension persisted to pH 7.0. If it is assumed that, as in a normal dissociation curve, the 50% point is 1 pH unit above the pH of 0.091 fractional dissociation (pH 7.5), then an estimate of pH 8.5 is obtained for the true pK_a. No comparable titratable group was ob-

[6] Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

served in II, indicating that the ionizable group detected in III is the primary ammonium one.

To detect sulfonamide ionization, a clear solution of III was quickly titrated to pH ~12 with NaOH. It remained soluble with a yellow fluorescence; upon back titration with HCl, a cloudy, blue fluorescing suspension emerged below pH 11.0. A similar yellow-blue transition was observed near "pH" 11.5 for II in 20% 1-propanol. No yellow fluorescing form of TNS was observed, even in 1.0 M NaOH, so it is assumed that the yellow-blue transitions of II and III characterize sulfonamide hydrogen ionizations.

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