Triazinylaniline Derivatives as Fluorescence Probes. Part 1. Absorption and Fluorescence in Organic Solvents and in Aqueous Media in Relation to Twisted Intramolecular Charge-transfer State Formation, Hydrogen Bonding, and Protic Equilibria

David J. Cowley,*,[†] Eddie O'Kane and Richard S. J. Todd
Department of Applied Physical Sciences, The University of Ulster, Coleraine, Northern Ireland, BT52 1SA, UK

The UV absorption and fluorescence characteristics of new optical probes based on a 1,3,5-triazinylaniline chromophore and of general structure p-$Et_2NC_6H_4C_3N_3(Cl)NR^1R^2$ are reported. Increase in alkanol solvent polarity strongly quenches the fluorescence by a process attributed to TICT (twisted intramolecular charge-transfer) state formation with co-operative solvent relaxation (log k_{NR}/s^{-1} 8.5–10.5). In aqueous media no changes in fluorescence band position or intensity are observed in the pH range 3.5 to 11 but below pH 3.5 protonation of the anilino nitrogen (pK_a *ca.* 2.4) eliminates both the λ = 360 nm absorption band and the weak λ = 460 nm fluorescence band. Free and hydrogen-bonded species, with respect to the anilino nitrogen centre, are present in aqueous dioxane solutions.

The triazinylaniline derivatives are prototypes of promising sensitive probes for organized media and as models of proteins labelled with the triazinylaniline fluorophore.

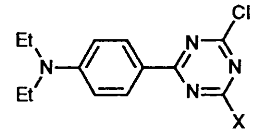
Extrinsic optical probe molecules[1–3] have been widely used to study molecular organization and dynamics in macromolecules and organized assemblies such as proteins[3,4] and lipid bilayers[2,5] since they can provide sensitive reporting of the effective polarity of the microenvironment and the constraints and timescales of molecular mobility.

The design of useful fluorescence probes[6,7] aims to produce a sensitive reporter with minimal structural perturbation of the probes system by way of structural compatibility and low dopant concentrations. Thus the meeting of the following general criteria is most desirable: (i) high molar extinction coefficient of near UV–VIS absorption; (ii) high quantum yield of fluorescence, *in situ*; (iii) fluorescence parameters responsive to site polarity; (iv) large Stokes' shift between absorption and emission; (v) emission from a well-defined electronic state in all media; (vi) well understood photophysics; (vii) low susceptibility to photobleaching.

If information on molecular movement is to be gained from fluorescence anisotropy data[8] then the satisfaction of further criteria is desirable. The intrinsic anisotropy in the absence of probe motion, r_0, should be high and an unambiguous, solvent invariant, relationship should exist between the absorption/emission transition dipole axes and a distinctive molecular axis. A single exponential decay of fluorescence, at least in homogeneous media, comparable to the timescale of environmental motions under investigation is highly desirable. Physicochemical considerations dictate that probes should not be abnormally bulky and that they should partition favourably into the phase or region of interest preferably with a restricted number or range of sites/orientations.

Use of a series of related probes may provide more reliable and informative data for interpretation at the detailed molecular level. Thus an aspect of the strategy of fluorescent probe design must be to incorporate opportunities for ready chemical variation and functionality whilst retaining a common fluorophoric system. We report here on a new series of triazinylaniline probes which conform well to the above criteria. They can be prepared by a facile nucleophilic displacement of chlorine[9] from the parent reactive dye TA–Cl, N,N-diethyl-4-(dichloro-1,3,5-triazinyl)aniline, under mild conditions:

$$Et_2NC_6H_4C_3N_3Cl_2 + Nu \longrightarrow Et_2NC_6H_4C_3N_3(Cl)Nu + HCl$$



X	Designation
Cl	TA-Cl
N(Me)2	TANMe2
N(Me)Bu^n	TAN(Me)Bu
N(Me)(n-octadecyl)	TANMOD

General formula of TA derivatives

Choice of nucleophile *e.g.* alkoxide, primary or secondary aliphatic amine *etc.* determines the functionality of the 'tail' for control of the physicochemical behaviour of the probe whilst retaining a common electronic fluorophore to all practical purposes. These probes are models also for the covalently bound products on labelling proteins with the reactive TA-Cl dye.

Compounds possessing this triazinylaniline chromophore $Et_2NC_6H_4C_3N_3(Cl)X$, where X = OR, NR^1R^2, show an intense absorption at λ = 360–370 nm and a strong fluorescence in low polarity solvents around λ 400–420 nm which arise from an intramolecular charge-transfer (ICT) transition. The role of solvent dipole reorientation in influencing the non-radiative decay of charge transfer excited states is a matter of current interest, thus the results reported here are discussed in that context in addition to their importance in fluorescence probe characterization.

[†] *Present address:* Marion Merrell Dow Research Centre, 16 rue d'Ankara, B.P. 447 R/9, 67009 Strasbourg Cedex, France.

Experimental

Materials.—Syntheses. TA–Cl was synthesized and purified by an adaptation of the method of Shaw and Ward.[10] Cyanuric chloride was recrystallized twice from dry redistilled CCl_4 and then sublimed (95 °C, 0.01 Torr*). *N,N*-Diethylaniline was redistilled at reduced pressure (93 °C, 6 Torr). A mixture of *N,N*-diethylaniline (27 g, 0.2 mol) and cyanuric chloride (18.4 g, 0.1 mol) was heated at 80 °C for 5 h under a slow stream of dry N_2. After the reaction had ceased the mixture was extracted with hot chloroform (200 cm^3) and the white crystalline hydrochloride salt of *N,N*-diethylaniline removed by filtration. Slow cooling and evaporation of the chloroform extract to a volume of *ca.* 50 cm^3 yielded good crystals of the TA–Cl product free of the colourless major reaction product *N*-(dichloro-1,3,5-triazinyl)-*N*-ethylaniline. Further purification of TA–Cl was by recrystallization from chloroform (yield 7.3 g, 25%).

Amine derivatives of TA–Cl, of general formula $Et_2C_6H_4C_3N_3(Cl)NR^1R^2$ were prepared[11] by dropwise addition of a solution of the appropriate amine R^1R^2NH (0.2 mmol amine in 5 cm^3) into a solution of TA–Cl in chloroform (29 mg, 0.1 mmol, in 10 cm^3) at 20 °C. The course of the reaction can be monitored spectrophotometrically since the absorption maxima shifts from λ = 400 nm to λ *ca.* 365 nm with an isosbestic point λ *ca.* 380 nm. After 20 to 60 min the solution was filtered to remove amine hydrochloride salt, and the chloroform removed by rotary evaporation. Subsequent purification was by recrystallization *e.g.* from light petroleum (b.p. 40–60 °C), or by TLC on silica gel with *e.g.* 3:1 v.v. ethyl acetate–light petroleum (b.p. 40–60 °C) eluent. The reaction can be performed conveniently also in acetone or diethyl ether at 5–25 °C. At higher temperatures with more reactive secondary aliphatic amines disubstitution of TA–Cl can occur to give $Et_2C_6H_4C_3N_3(NR^1R^2)_2$, whose absorption maxima lie at λ 335–345 nm.

Analytical and Spectroscopic Data.—Compounds of general formula *p*-$Et_2C_6H_4C_3N_3(Cl)X$.

(i) TA–Cl, X = Cl: m.p. 156 °C (from chloroform) (lit.,[10] 157 °C) (Found: C, 52.7; H, 4.7; N, 19.0; Cl, 24.1%; M^+ 296.0578. $C_{13}H_{13}Cl_2N_4$ requires C, 52.54; H, 4.75; N, 18.85; Cl, 23.9%; *M*, 296.0586). δ_H(60 MHz; $CDCl_3$, standard Me_4Si) 1.24 [6 H, t, *J*† 7, $N(CH_2CH_3)_2$], 3.52 (4 H, q, *J* 7, NEt_2), 6.72 (2 H, d, *J* 9, arom H *ortho* to NEt_2) and 8.37 (2 H, d, *J* 9, arom H *meta* to NEt_2).

(ii) $TANMe_2$, X = NMe_2: m.p. 118–119 °C [from light petroleum (b.p. 60–80 °C)] (lit.,[11] 118 °C); (Found: C, 58.9; H, 6.55; N, 23.0; Cl, 12.2. $C_{15}H_{20}ClN_5$ requires C, 58.94; H, 6.60; N, 22.9; Cl, 11.6%) δ_H(60 MHz; $CDCl_3$ at 35 °C; standard Me_4Si) 1.19 (6 H, t, *J* 8, NEt_2), 3.25 (6 H, br s, NMe_2, restricted rotation about ring–NMe_2 bond), 3.43 (4 H, q, *J* 8, NEt_2), 6.68 (2 H, d, *J* 8, arom H *ortho* to NEt_2) and 8.32 (2 H, d, *J* 8, arom H *meta* to NEt_2).

(iii) TAN(Me)Bu, X = NMeBu: m.p. (glassy solid from CH_2Cl_2) (Found: C, 62.75; H, 7.8; N, 20.4; Cl, 9.1%; M^+ 347.1895. $C_{18}H_{26}ClN_5$ requires C, 62.15; H, 7.5; N, 20.1; Cl, 10.2%; *M*, 347.1877.) δ_H(60 MHz, $CDCl_3$ at 35 °C, standard Me_4Si) 1.21 (6 H, t, *J* 8, NEt_2), 1.0–1.8 [7 H, m, $N(CH_2)_2CH_3$], 3.20 and 3.28 (1.5 H each, s, NMe two rotational isomers about the triazine ring–N(Me)Bu bond), 3.46 (4 H, q, *J* 8, NEt_2), 3.65 (2 H, br t, *J* 8, $NCH_2C_3H_7$, rotational isomers), 6.65 (2 H, d, *J* 8.4, arom H *ortho* to NEt_2) and 8.25 (2 H, d, *J* 8.4, arom H *meta* to NEt_2).

(iv) TANMOD, X = $N(Me)(C_{18}H_{37})$: m.p. 35–36 °C (from chloroform) (Found: C, 70.72; H, 9.85; N, 12.6; Cl, 6.4; M^+ 543.3964. $C_{32}H_{54}ClN_5$ requires C, 70.6; H, 10.0; N, 12.9; Cl, 6.5%; *M*, 543.4068) δ_H(60 MHz, $CDCl_3$ at 35 °C standard Me_4Si) 1.1–1.4 (41 H, m, NEt_2 and $CH_2C_{17}H_{35}$), 3.21 and 3.30 [1.5 H each, s, NMe, two isomers by restricted rotation about the triazine ring–$N(Me)(C_{18}H_{37})$ bond], 3.48 (4 H, q, *J* 7, NEt_2), 3.78 (2 H, br t, $NCH_2C_{17}H_{35}$), 6.70 (2 H, d, *J* 9, arom H *ortho* to NEt_2) and 8.32 (2 H, d, *J* 9, arom H *meta* to NEt_2).

Optical Measurements.—UV absorption spectra were measured on Unicam SP8000A and Perkin-Elmer Lambda 5 instruments. Protonation of TA derivatives was monitored spectrophotometrically. Aliquots of a stock solution of TAN compound in acetonitrile (typically 100 mm^3 of 1 mmol dm^{-3} solution) were dispersed into aqueous buffer containing 25% v/v ethanol (2.5 cm^3) and of known pH, measured on a Phillips PW9410 meter at 20 °C. Hydrochloric acid was used to cover the pH range 0–4, acetate buffer pH 4–6, citrate buffer pH 6–7.5, tris buffer pH 7.5–9.6, carbonate–bicarbonate solutions pH 9.6–11.5 and sodium hydroxide for pH > 11.

Fluorescence spectra were measured on a Perkin-Elmer MPF-44B spectrofluorimeter for optically dilute solutions (absorbance < 0.10/10 mm path) in 10 mm silica cuvettes without deoxygenation. Quantum yields of the TA dyes in toluene were determined relative to quinine bisulphate in 0.5 mol dm^{-3} sulphuric acid (Φ_F 0.54) under λ = 350 nm excitation, and these values used to determine quantum yields for the dyes in spectroscopic grade alcoholic solvents for λ = 355–370 nm excitation wavelengths at 4 nm bandpass. In all cases correction for solvent refractive index differences were made. Fluorescence decay times were obtained using an Applied Photophysics pulse sampling system with an Edinburgh Instruments nanosecond flashlamp source operating on nitrogen gas at 1 atm. Signal output as a function of ramp voltage was collected on a BBC model B microcomputer. Deconvolution of excitation and sample emission profiles was performed by the Phase Plane method[12] to derive a single-exponential lifetime. All samples gave satisfactory fits on this simple analysis; the validity of this assumption was confirmed subsequently by comprehensive single photon counting experiments on selected samples using the Daresbury synchrotron radiation facility.

Results and Discussion

Absorption and Fluorescence of TA Systems.—Band energies. The lowest energy absorption band of all TA derivatives corresponds to an intramolecular charge-transfer (ICT) $\pi^* \longleftarrow \pi$ transition of high oscillator strength (Table 1). This ICT transition corresponds electronically to an appreciable transfer of charge from the highest occupied molecular orbital embracing the benzene ring and the exocyclic (anilino) nitrogen, which together constitute the donor D, to the lowest unoccupied molecular orbital located here essentially on the acceptor triazinyl ring system.[13,14]

Replacement of a single chlorine of TA–Cl by a conjugating electron donor substituent lowers the π electron acceptor character of the triazine ring, thereby raising the LUMO energy and shifting the absorption–fluorescence band strongly to the blue, by about λ = 30 nm in the case of an amine substituent group NR^1R^2. The fluorescence band position is more sensitive to the polarity of the solvent than is the absorption band position (see Tables 2 and 3).

Ooshika–Lippert–Mataga plots[15] of Stokes' shift, $\bar{\nu}_A - \bar{\nu}_F$, *versus* the solvent polarity function $f(n,\varepsilon_r)$, where $f(n,\varepsilon_r) = (\varepsilon_r - 1)/(2\varepsilon_r + 1) - (n^2 - 1)/(2n^2 + 1)$ are linear (Fig. 1). The calculated increases in electric dipole moment on ICT excitation for TA–Cl, $TANMe_2$ and TANMOD are 23.7, 17.7

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* 1 Torr = 133.32 Pa.

† *J* values are given in Hz throughout.

Table 1 UV Absorption characteristics of triazinylanilines, TA–X, in methanol at 20 °C

Compound	Molar extinction coefficient[a] $\varepsilon/\mathrm{dm^3\,mol^{-1}\,cm^{-1}}$	Band maxima λ/nm	Oscillator strength f	$k_{SB}/10^8\,s^{-1}$[b]
TA–Cl	50 000	400	0.66	2.75
$TANMe_2$	40 730	360	0.66	3.39
TAN(Me)Bu	41 800	361	0.68	3.48
TANMOD	42 210	360	0.66	3.40

[a] Molar extinction coefficients accurate to ±100 $\mathrm{dm^3\,mol^{-1}\,cm^{-1}}$, band maxima to ±1 nm. [b] k_{SB} is the radiative rate constant estimated from the Strickler–Berg expression on the basis of the absorption band characteristics.

Table 2 Fluorescence lifetimes and band positions of some triazinylaniline derivatives in toluene and alkanol solvents at 20 °C

		$TANMe_2$		TAN(Me)Bu	TANMOD
Solvent	ε_r[a]	λ_F/nm	τ_F/ns[b]	τ_F/ns[b]	τ_F/ns[b]
Toluene	2.4	391	1.42	1.07	1.53
Decan-1-ol	8.1	412	1.40	1.10	1.38
Octan-1-ol	10.3	414	0.90	0.91	1.20
Hexan-1-ol	13.3	416	0.77	0.70	0.82
Butan-1-ol	17.5	418	0.60	0.31	0.55
Propan-1-ol	20.3	419	0.47	—	0.49
Ethanol	24.5	419	0.31	(0.12)	0.44

[a] ε_r = relative permittivity of the solvent at 20 °C. [b] The fluorescence lifetimes for $TANMe_2$ and TANMOD were determined by pulse sampling fluorimetry and are reckoned to be accurate to ±0.10 ns. In the case of TAN(Me)Bu the lifetimes given were determined using synchrotron radiation as excitation impulse with conventional nanosecond single photon counting detection, and are thereby more reliable; but close agreement with the lifetimes obtained by sampling fluorimetry was found in all cases. The wavelengths of fluorescence of TAN(Me)Bu and TANMOD parallel closely those of $TANMe_2$ and are omitted from the table.

Table 3 Variation of fluorescence intensity and band position of TAN(Me)Bu in aqueous dioxane mixtures at 20 °C

% v/v Dioxane in water	Fluorescence band maxima λ/nm	Relative yield of fluorescence
100	402	100.0
90	413	21.3
80	416	7.8
70	420	4.2
50	431	0.6
40	430	0.9
30	425	1.5
20	(broad top)	1.8
10	(broad top)	2.0
0	459, shoulder at λ 410 nm	2.2

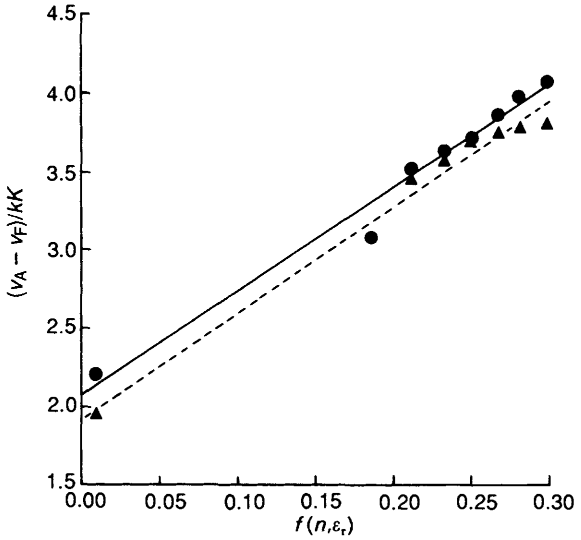

Fig. 1 Ooshika–Lippert–Mataga plot of the difference in wavenumber of absorption and fluorescence maxima *versus* solvent polarity function $f(\varepsilon_r,n)$ for $TANMe_2$ (circles) and TANMOD (triangles) where $f(\varepsilon_r,n) = (\varepsilon_r - 1)/(2\varepsilon_r + 1) - (n^2 - 1)/(2n^2 + 1)$ and ε_r is the relative permittivity and n is the refractive index of the solvent.

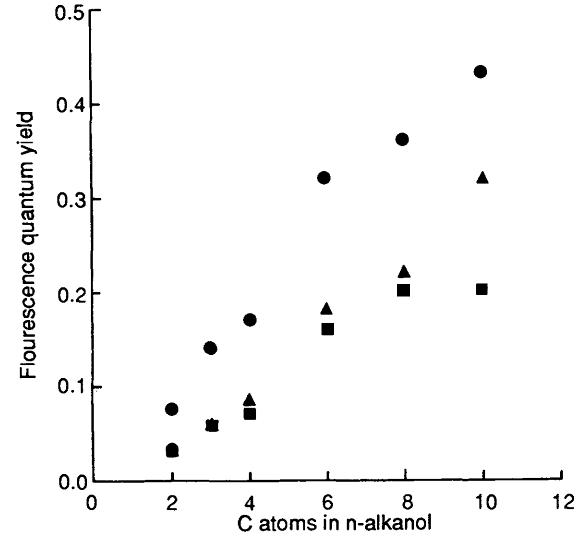

Fig. 2 Fluorescence quantum yields of $TANMe_2$ (triangles), TAN(Me)Bu (squares) and TANMOD (circles) in alkanols at 20 °C

and 18.1 Debye, respectively, based on effective molecular volumes a^3 containing the electric dipole of the TA chromophore of 0.49 and 0.56 $\mathrm{nm^3}$ for TA–Cl and $TANMe_2$, respectively, whilst the effective volume of the solvated chromophore in TANMOD should be very close to that of $TANMe_2$. The values confirm the strong ICT character of the transition and the reduction in electron acceptor ability of the triazine ring on amino substitution of a chlorine.

Fluorescence Quantum Yields and Lifetimes. (a) *Solvent polarity effects.* The fluorescence quantum yields and lifetimes measured for amino-substituted TA compounds in alkanol solvents decline smoothly with increase in solvent polarity (see Table 2 and Fig. 2). The radiative rate constant k_R declines slowly with increase in solvent polarity, as judged by the relative permittivity in the series of alkanol solvents, and agrees well at low polarity with the Strickler–Berg estimates (Table 1) of *ca.* $3.4 \times 10^8\,s^{-1}$, $\log k_R = 8.53$ (compare Fig. 3). The non-radiative decay constant k_{NR} is strongly enhanced by increase in solvent polarity (Fig. 3) but the effect is much greater for the parent TA–Cl than for $TANMe_2$, the latter being typical of the amino-substituted derivatives. A correlation between k_{NR} and solvent viscosity appears to exist (Fig. 4) for the alkanol solvents,

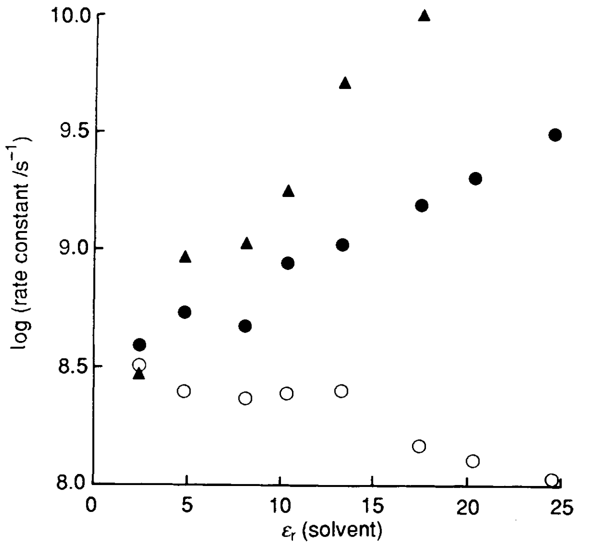

Fig. 3 Logarithms of the rate constants of non-radiative (filled circles) and radiative (open circles) decay of the fluorescent state of TANMe$_2$ in alkanols and other organic solvents as a function of solvent relative permittivity. Logarithms of the non-radiative rate constants for the parent dye TA–Cl (filled triangles) are shown for comparison (taken from ref. 13).

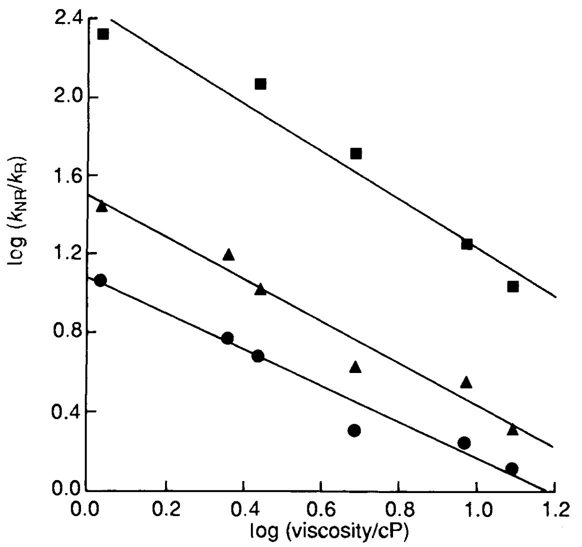

Fig. 4 Correlation of log (k_{NR}/k_R), *i.e.* log (Φ^{-1} − 1), with log (viscosity) for TA derivatives in alkanol solvents at 20 °C. The best linear fits give slopes of − 1.24 ± 0.15 (TA–Cl, squares), −0.90 ± 0.09 (TANMe$_2$, circles) and − 1.07 ± 0.11 (TANMOD, triangles).

and suggests an interplay between molecular motions of the solute dye and the solvent dipoles in the deactivation of the ICT excited states of these compounds. The main emitting species of the TA–X compounds has high, but not maximum, charge separation as shown by the sensitivity of the fluorescence band position *etc.* to the external solvent environment. It has been argued[13] that, together with analogous R$_2$NArA systems,[16,17] the initial and fluorescent excited state of the TA–Cl dye has a coplanarity of the anilino nitrogen and the benzene ring *i.e.* full conjugation and that the major deactivation pathway involves a twisting away from coplanarity with a further enhancement of the electronic charge separation to yield a TICT (twisted intramolecular charge-transfer) state. The TICT state itself may exhibit a weak fluorescence strongly red-shifted with respect to the emission of the precursor ICT state and generally longer lived.[13,16] In the present work the presence of the product TICT was not detectable in the observed fluorescences.

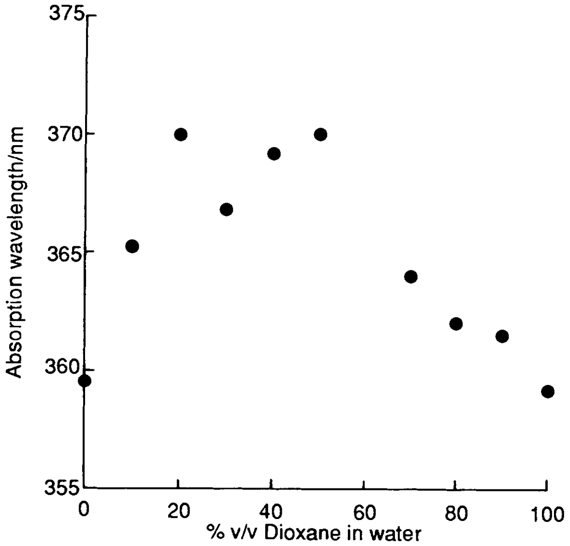

Fig. 5 Absorption band maxima of TAN(Me)Bu in dioxane–water mixtures at 20 °C

(b) *Role of solvent dipole relaxation.* The possible roles played by the internal molecular rotation[18] in the *N*,*N*-diethylanilino donor moiety and the relaxational movement of solvent dipoles[19–24] in determining the rate of the solute nonradiative decay to TICT states are of some current interest. In the case of the parent TA–Cl the nonradiative decay time of *ca.* 100 ps in butanol is close to, but still larger than, the relevant longitudinal dielectric relaxation time τ_d' of the alcohol[25] (72 ps) whereas in the same solvent the fluorescence decay time of, for example, TANMe$_2$, is 600 ps. Thus for the (mono)amino-substituted TA derivatives the process of deactivation of the first excited state, by an internal linear charge flow linked to twist about the Et$_2$N–benzene ring bond, is not limited by the solvent dipole relaxation rate *per se*. Since the deactivation rates of TA–Cl and TANMe$_2$ in the same solvent differ considerably, despite the common donor component Et$_2$N, the direct viscous resistance of the solvent impeding the donor group rotation does not appear to be the main limiting factor.[21] Rather an electronic barrier of diminishing size with increasing acceptor strength of the triazine ring π system and/or increasing solvent dipolarity must exist. Following the Marcus approach the overall barrier to decay (deactivation of the primary fluorescent state to a TICT state here) is the sum of an intrinsic electronic activation energy and a solvent reorganizational energy relating to the charge separation change in the solute.[22] For a given TA fluorophore the observed linear correlation of log (k_{NR}/k_R) and log (solvent viscosity) with near unity gradient must arise from the solvent re-organizational term (*cf.* ref. 20). The processes of making and breaking hydrogen bonding structures in the alkanol solvent responsible for the bulk viscosity behaviour might well correspond to the rotation of the solvent electric dipoles in a dielectric relaxation formalism[23] — hence the apparent relationship noted above.

Absorption and Fluorescence in Aqueous Media.—Hydrogen bonding equilibria. Since the TA derivatives are potentially useful as probes of structure in organized media or of proteins in the presence of water, the effects of water on the probe emission characteristics were evaluated. Examination of dioxane–water solutions provided a key to the elucidation of complex and subtle effects in the observed spectra of aqueous solutions.

Measurements were performed mainly on TAN(Me)Bu as representative of the present set of TA derivatives.

The position of its ICT absorption band (Fig. 5) attains a maximum value at around 50% v/v water in dioxane. From 0 to 50% v/v water in dioxane the expected red shift of an ICT transition is observed. Extrapolation of this normal trend

Table 4 p*K*a values of some electron acceptor *para*-substituted *N*,*N*-dialkylanilines in 25% v/v ethanol in aqueous buffers at 20 °C

Compound[a]	pK_a	UV absorption maxima, λ/nm
TA–NMe_2	2.45	363
TA–NH_2	2.00	375
TA–N(Me)Bu	2.42[b]	362
TA–OEt	2.20	362
TA–OMe	2.10	362
TA–Cl	0.60	407
p-$Me_2NC_6H_4CN$	0.18	293
p-$Me_2NC_6H_4NO_2$	0.25	400

[a] TA = *p*-$Et_2NC_6H_4C_3N_3(Cl)$–. [b] Determined in 50% v/v aqueous dioxane.

would predict the absorption maxima to lie at λ = 380 nm in water alone as solvent; experimentally the band maxima is at λ = 360 nm — lower than in 50–50 v/v water–dioxane solution.

This blue shift of the absorption band *i.e.* raising of the energy of the ICT transition, is attributed to a reduction in the capacity of the Et_2N group to release charge into the aromatic ring due to participation of the formal lone pair on the tertiary anilino nitrogen in specific hydrogen bonding to water.[27] In water this specific complexation is most probably complete. (Note that hydrogen bonding or indeed protonation of a triazine ring nitrogen centre would be expected to create a small red shift of the ICT band —contrary to observation). Similar effects have been reckoned as important for TICT state formation in *p*-$Me_2NC_6H_4CN$.[26]

The fluorescence band of TAN(Me)Bu in aqueous dioxane behaves similarly to the absorption band (Table 3). A progressive red shift is found with water content increasing from 0 to 50% v/v and the band width at half maximum intensity, *w*, only increases from 40 to 52 nm. Thereafter *w* increases markedly, reaching 112 nm at 90% v/v water content, until in water as solvent a distinctive shoulder at λ =410 nm is seen on the main band whose maxima lies at λ = 459 nm. The short wavelength shoulder is consistent with emission from a species having specific hydrogen bonding to water *via* the anilino nitrogen centre. The λ = 459 nm emission band is presumed to arise from free (*i.e.* non-specifically complexed) excited dye produced by dissociation, following light excitation, of the specifically complexed species which dominates the grand state dye population in water. The fluorescence intensity at the band maxima shows the expected dramatic fall on progressive addition of water (0 to 50% v/v) to dioxane solutions of TAN(Me)Bu (Table 3). However, further increase in water content leads to a small increase in overall intensity of fluorescence, consistent with the hydrogen bonding model. TA dye to which water is directly hydrogen bonded at the anilino nitrogen will be less quenched as a result of the general polarity of the solvent microenvironment than the 'free' TA dye species, just as TAN derivatives having excited states of lower ICT character suffer less quenching of fluorescence than the parent TA–Cl. Thus as the 'free' TAN(Me)Bu is gradually converted into the 'hydrogen bonded' form at the higher water concentrations so the fluorescence intensity rises (despite further overall polarity increase).

Alternative explanations invoking protonation equilibria are possible in principle but can be eliminated on the following grounds.

Effects of protonation equilibria (*a*) *Electronic ground state of* TAN *systems*. Over the pH range 3.5–11 in highly aqueous solutions the absorption band maxima is invariant at λ = 362.1 ± 0.9 nm as are both the fluorescence band structure (main peak at λ = 460 nm and a shoulder at λ = 410 nm) and the intensity.

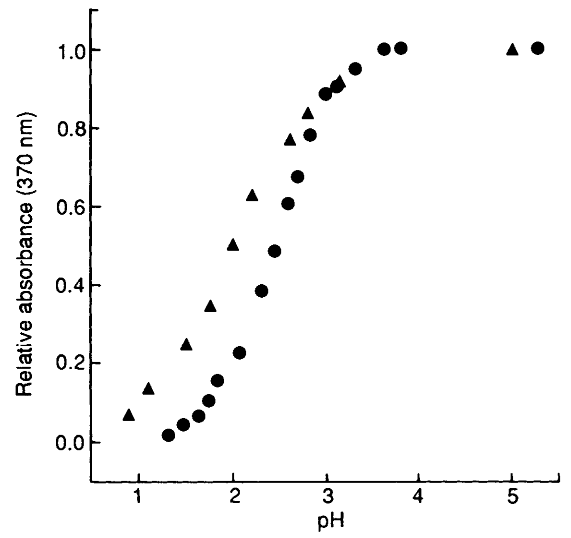

Fig. 6 Effect of pH on the intensity of the ICT absorption band at 20 °C for TAN(Me)Bu in 50% v/v aqueous dioxane (circles) and $TANH_2$ in 75% v/v aqueous ethanol (triangles)

The spectroscopic consequences of protonation at the three classes of nitrogen atom in the triazinylaniline derivatives are predictable. Protonation at the anilino nitrogen centre, the major donor component of the TA chromophore, leads to a loss of the intense ICT absorption band at λ = 367 nm which is shifted to λ = 252 nm. This large change is readily monitored and was used to determine the acid dissociation constant (pK_a) of the conjugate acid of a number of TA derivatives and related donor–acceptor compounds. Representative titration curves are shown in Fig. 6. The derived pK_a values (Table 4) follow the expected trends in relation to the variation in electron acceptor character with constant donor component. Thus the pK_a of TA–Cl is six units lower than that of *N*,*N*-diethylaniline and similar to that of *N*,*N*-dialkylanilines bearing a nitrile or nitro group in the *para* position. Substitution of one chlorine in TA–Cl by an alkylamino group increases the pK_a by two units.

If, hypothetically, protonation of the nitrogen atom exocyclic to the triazine ring (type *c*) were to occur at a higher pH than that of the anilino nitrogen (type *a*) then, at least for a short range above pH 3, a considerable red shift of the ICT transition band should be observable since the protonated amino substituent would behave akin to chlorine in the parent TA–Cl compound. Such was not found in practice. The effects of prior protonation of an aza nitrogen in the triazine ring (type *b*) would be similar in direction but smaller in magnitude to type *c* protonation. Again, despite a careful search such perturbations were absent from the experimental spectra. Intercomparison of published data on pK_a values[28–32] in azaaromatics and Hammett relationships allowed estimation of the pK_as expected for *e.g.* $TANMe_2$, as follows:

Type *b*, pK_a +0.75–1.75
Type *c*, $pK_a < -6$

The pK_a for type *b* protonation will be lowered (to −1.25?) in the likely situation of prior type *a* protonation. Thus for the electronic ground state of TAN derivatives only protonation of the anilino nitrogen can assume any importance in ordinary aqueous solutions.

(*b*) *Excited state of* TAN *systems*. Since electronic excitation may alter acidity/basicity by several orders of magnitude it was necessary also to examine the fluorescence spectra of the TA derivatives as a function of pH over a wide range.

Experimentally the form and intensity of the fluorescence spectrum of TAN(Me)Bu at constant concentration in buffered aqueous media were independent of pH from pH 11 to *ca.* 3.5.

Below pH 3.5 the intensity of the fluorescence diminished in a manner analogous to that of the UV absorption intensity from which a pK_a of 2.42 (in 10% v/v acetonitrile in water) was derived, identical to that for the ground state pK_a (see Table 4). Similar results were obtained for TAN(Me)Bu in 50% v/v dioxane in aqueous buffer for which the fluorescence peak remained constant at λ 425 nm.

The absence of excited-state prototropic equilibrium has been observed also in the case of 1-(9-anthryl)-*N*,*N*-dimethylaniline[33] in ethanol–water mixtures.

Since ICT excitation reduces the availability of electrons at the anilino nitrogen the excited state pK_a* for type *a* protonation will be much lower than 2.5 (pK_a of ground state) and thereby not influence the fluorescence in the aqueous solutions at normal pH values. Transfer of charge on excitation into the triazine ring could increase the apparent pK_a* for the aza nitrogen(s) from pK_a (type *b*) of -1.25 into the region below $+4$. However, no changes in spectral shape or in the position of the band maximum were observable for TAN(Me)Bu solutions over the pH range 4 to 2, as the overall intensity of the fluorescence band dropped towards zero.

Conclusions

The photophysical properties of the triazinylaniline derivatives reported here and their sensitivity of response to solvent microenvironment satisfy the desirable characteristics and criteria for good optical probes as summarized in the Introduction section.

The large increase in fluorescence yield with increase in hydrophobicity of environment and the invariance with pH over a wide range of the weak fluorescence in aqueous solution make these fluorophores attractive as probes of organized media. The relative ease and mode of substitution of the parent TA–Cl dye under chemically mild conditions make this a potentially useful precursor in fluorescent probe design for studies of bilayer membrane systems and also in selective labelling of proteins and peptides. Studies are in progress on both of these aspects.

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