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Influence of Reverse Micellar Environments on the Fluorescence Emission Properties of Tryptophan Octyl Ester

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A number of recent studies have presented perspectives on the hydrophobic fluorescence probe tryptophan octyl ester (TOE). This molecule has attracted notable attention as a suitable model for the natural fluorophore tryptophan, in case of membrane proteins. We report here, for the first time, the fluorescence emission behaviour of TOE in reverse micelles of aerosol-OT (AOT) in *n*-heptane, containing different amounts of water. Relevant studies in representative homogeneous solvent media are also included for comparison. The fluorescence emission parameters (especially emission maximum, relative intensity, and anisotropy) of TOE are found to exhibit significant variation upon changes in the water/surfactant molar ratio (w_0) of the reverse micelles. Fluorescence decay studies on TOE which we have also performed, indicate biexponential decay kinetics in reverse micelles as well as in homogeneous solvent media. The implications of these findings are examined in relation to the potentialities of TOE as a novel fluorescence probe for membrane proteins present in water restricted environments prevailing at the interfaces of biomembranes (for which reverse micelles serve as ideal model systems).

Key Words: **tryptophan octyl ester; fluorescence probe; membrane proteins; AOT reverse micelles; water restricted environments; fluorescence anisotropy; fluorescence lifetime.**

The role of tryptophan residues in the structure and function of membrane proteins has attracted significant recent attention (1). Because of its aromaticity, the tryptophan is capable of π–π interactions and weakly polar interactions (2). In the context of protein optical spectroscopy, tryptophan is especially important, since it is the principal source of intrinsic fluorescence of most proteins. Therefore, traditionally tryptophan has been the common and obvious choice as an optical probe in fluorescence spectroscopic studies of proteins, in general (3–5). However, the analysis of fluorescence from proteins containing multitryptophan residues is often complicated because of the complexity of the fluorescence emission parameters (viz. lifetime, quantum yield) resulting from environmental sensitivity of individual tryptophan residues (1, 6). Use of appropriate model fluorophores, which can be viable alternatives to the natural fluorophore, tryptophan, should be helpful in such cases. In view of this, some years ago, a number of tryptophan analogs, e.g., 7-azatryptophan (7AT) and 5-hydroxytryptophan (5HT) were proposed as intrinsic probes for the study of protein structure, function and dynamics (7, 8). Various workers, including our group, have reported extensive studies on different aspects of the photophysics of these fluorophores and their respective chromophoric moieties (7–11). However, it is being increasingly recognized that the hydrophobic fluorophore tryptophan octyl ester (TOE, structure shown in Fig. 1 inset) is a more suitable model for tryptophan, in case of membrane proteins (12–17). Thus it is of considerable importance to carry out detailed characterization of the fluorescence emission properties of TOE in appropriate environments relevant to biomembranes.

In connection with studies related to biomembranes, it is often convenient to use simple model systems, which can mimic at least some of the important and essential physico-chemical features of the membrane architecture and related aspects, and at the same time, lack much of the complexities of natural membranes. Typical examples of such membrane mimetic models are liposomes, and normal as well as reverse micelles. Liposomes are simply vesicles carrying an aqueous volume bound entirely by a membrane made up of lipid molecules (18). Normal and reverse micelles are organized assemblies of surfactants in aqueous and organic solvent media respectively (8, 19, 20). Ladokhin and Holloway (15) have examined TOE as a model for tryp-

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tophan fluorescence from proteins in model (liposomal) membrane environments. They observed that the depth dependent fluorescence quenching of TOE by brominated lipids proceed via a dynamic mechanism. They further showed that with the quenchers attached to specific positions along the lipid acyl chains, one can obtain vital structural information on membrane bound peptides and proteins. Chattopadhyay *et al.* (16) have characterized the ionization properties of this fluorophore (TOE) in model (liposomal) membranes of dioleoyl-sn-glycero-3-phosphocholine (DOPC). In particular, they investigated its partitioning and motional characteristics in such membranes as a function of its ionization state. More recently, Foresta *et al.* (12) investigated the fluorescence properties of TOE in various mixed micellar systems of dodecylmaltoside (DM) and 7,8-dibromododecyl β-maltoside (BrDM) or 10,11-dibromoundecanoyl β-maltoside (BrUM). They found that the indole moiety of TOE is highly accessible to the bromine atoms located on the detergent alkyl chain because quenching by bromine occurs by direct contact with the fluorophore.

In the present communication, we describe preliminary findings on the fluorescence emission properties on TOE in reverse micellar solutions of aerosol-OT (AOT) in *n*-heptane, containing various amounts of H_2O. Relevant data, from studies on solutions in representative homogeneous solvent media, are also included herein for comparison. Reverse micelles, in particular, afford the opportunity of examining molecules with various states of hydration, simulating situations present in water restricted environments prevailing in membrane interfaces. The extent of hydration in reverse micellar assemblies can be conveniently modulated by gradual addition of water to the surfactant solutions (in organic solvents) and thereby varying the water/surfactant molar ratio (w_0) (7, 9, 19, 20).

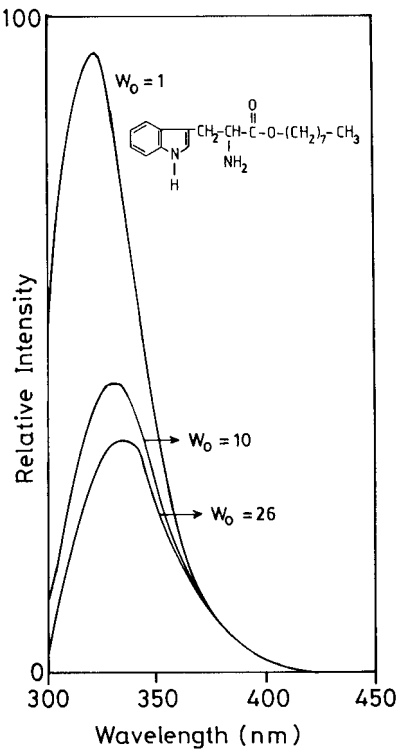


FIG. 1. Fluorescence emission spectra of TOE in AOT/*n*-heptane reverse micelles at various values of w_0. λ_{ex} = 290 nm, excitation bandwidth = 3 nm, emission bandwidth = 5 nm. Inset: Structure of TOE.

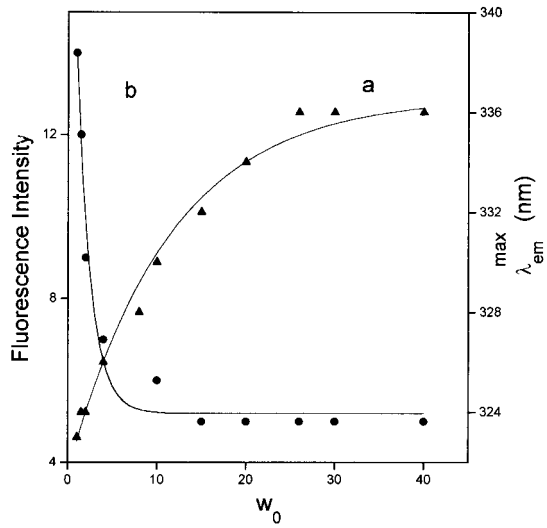


FIG. 2. Effect of H_2O addition on the (a) fluorescence λ_{em}^{max} and (b) relative fluorescence intensity of TOE in AOT/*n*-heptane reverse micelles.

In the present work we observe that at very low water content (w_0 = 1), the fluorescence emission maximum (λ_{em}^{max}) of TOE occurs at 323 nm which is significantly blue shifted relative to the emission peak in water (≈347 nm). With gradual addition of water to the reverse micellar solution, the emission maximum progressively shifts to higher wavelengths ($\lambda_{em}^{max} \approx$ 336 nm at w_0 = 40). Moreover, the red shift in the λ_{em}^{max} is accompanied by drastic changes in the relative fluorescence intensity, which is in marked contrast to the case of tryptophan where this particular parameter is rather insensitive to changes in w_0 (21). Furthermore, from the λ_{em}^{max} values observed, we have obtained quantitative estimates of the polarities (in terms of empirical polarity parameter $E_T(30)$ (22)) of the microenvironments of the TOE molecules in the reverse micelles, at different values of w_0. However, from the fluorescence emission maximum (λ_{em}^{max}), anisotropy (r) and lifetime(τ) data reported in this paper, it is apparent that through out the range of w_0 values (from low to high) the fluorophore (TOE) molecules reside in the interfacial region and do not enter the bulk water phase even at very high water contents (w_0 = 40). These results are interpreted in terms of modifications in the micro-

TABLE 1

Fluorescence Emission Parameters of TOE in Different Solvents

Solvents	$E_T(30)$[a]	λ_{em}^{max} (nm)	τ_1 (nsec)	A_1	τ_2 (nsec)	A_2	$\langle\tau\rangle$ (nsec)
Water	63.1	347	0.64	71.16	2.35	28.84	1.13
Methanol	55.5	342	1.82	88.50	5.00	11.50	2.18
Ethanol	51.9	337	2.52	74.79	1.13	25.21	2.17
Acetonitrile	46	334	4.76	83.33	0.81	16.67	4.10
Ethylacetate	38.1	329	2.87	65.26	0.82	34.74	2.16

[a] Values taken from Ref. 22.

environment of TOE upon variations in the water content (w_0) of the reverse micelles. The present findings highlight, for the first time, the advantages of TOE as a fluorescent probe for water restricted environments, with strong potential for applications to peptides and proteins occurring at the interfaces of biomembranes.

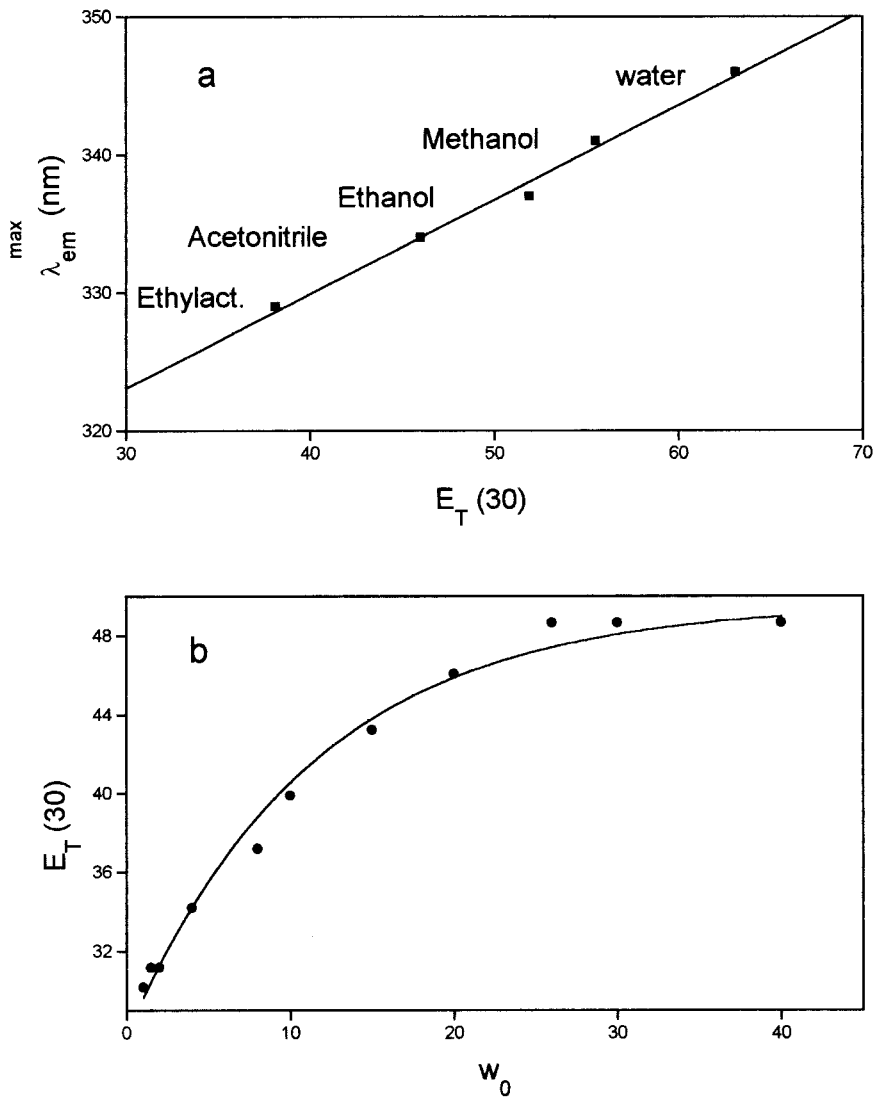


FIG. 3. (a) λ_{em}^{max} of TOE in different solvents versus $E_T(30)$ values for fluorescence emission. (b) $E_T(30)$ values (evaluated using calibration plot given above) versus w_o in AOT/*n*-heptane reverse micelles. λ_{ex} = 290 nm, excitation bandwidth = 3 nm, emission bandwidth = 5 nm.

EXPERIMENTAL DETAILS

TOE and sodium bis-(2-ethylhexyl)sulfosuccinate (AOT) were purchased from Aldrich Chemical Company and Sigma, respectively, and were used as received. Spectroscopic quality solvents from Merck were used without further purification, after confirming the absence of absorbing and fluorescent impurities. Concentrated stock solutions of TOE were prepared in triply distilled water. For work in reverse micelles, small aliquots from this stock solution were added to freshly prepared solutions of AOT (≈100 mM). Appropriate amounts of H_2O were subsequently added to make solutions having different water/surfactant molar ratio (w_o) values. The final fluorophore concentrations were kept around ≈16 μM in homogeneous as well as reverse micellar solutions. At such low fluorophore concentrations, not more than one TOE molecule would be present per reverse micelle on an average (9), which rules out significant probe aggregation effects.

Steady-state absorption and fluorescence emission measurements were carried out using Hitachi models U-2000 spectrophotometer and F-4010 spectrofluorometer respectively. Fluorescence spectra were in general corrected for the wavelength dependence of the sensitivity of the apparatus (23). In case of emission measurements of the probe in reverse micellar solutions, background fluorescence as well as light scattering from the AOT reverse micellar preparations were removed by subtraction of the spectra recorded on blank solutions. Fluorescence lifetime measurements were carried out using a nanosecond single photon counting apparatus, as described in a previous work (24). Data analysis was performed by a reconvolution method using a nonlinear least square fitting programme. The goodness of fit was estimated by χ^2 criteria and randomness of residuals and their autocorrelation (25, 26). Average lifetime ($\langle\iota\pm$) was evaluated from the equation: $\langle\tau\pm = (A_1\tau_1 + A_2\tau_2)/(A_1 + A_2)$, where A_1, A_2, and τ_1, τ_2 represent the amplitudes and time constants, respectively, of the individual components in the biexponential decay profiles. The fluorescence anisotropy (r) values were obtained using the expression $r = (I_{VV} - GI_{VH})/(I_{VV} + 2\,GI_{VH})$, where I_{VV} and I_{VH} are the vertically and horizontally polarized components of TOE emission at 340 nm, with excitation by vertically polarized light at 290 nm and G is the sensitivity factor of the detection system (23). Each intensity value used in this expression represents the computer averaged values of ten successive measurements. All spectroscopic measurements were carried out at room temperature (298K).

RESULTS AND DISCUSSION

The fluorescence emission spectra of TOE (~1.5×10^{-5} M) were examined in AOT/*n*-heptane reverse micelles containing different amounts of added water. Typical spectra are displayed in Fig. 1. At the lowest water content ($w_0 = 1$), the emission maximum (λ_{em}^{max}) occurs at ~323 nm. With increasing w_0, the spectrum undergoes progressive shifts to longer wavelengths, which is accompanied by dramatic decrease in the fluorescence intensity. Figure 2a (the curve shown by triangular points) displays the variation of λ_{em}^{max} as a function of w_0. Interestingly, the increase in λ_{em}^{max} with change in w_0 is extremely rapid up to $w_0 \approx 15$, beyond which the change is more gradual with λ_{em}^{max} eventually reaching 336 nm at $w_0 = 40$. In Fig. 2b, we present variation of relative fluorescence intensity as a function of w_0. It may be noted that the remarkable decrease in fluorescence intensity with increase in w_0, we observed here in case of TOE is in marked contrast to the case of tryptophan where the emission yield is relatively insensitive to changes in w_0 (21).

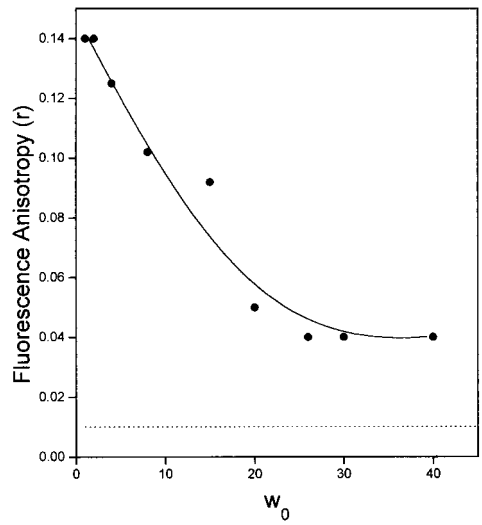


FIG. 4. Variation of fluorescence anisotropy (r) of TOE in AOT/*n*-heptane reverse micelles with increase in w_0. The dotted line indicates the fluorescence anisotropy of TOE in water. λ_{ex} = 290 nm, bandwidth = 3 nm, and λ_{em} = 340 nm, bandwidth = 5 nm.

In order to obtain quantitative measures of the polarity of the local environments of TOE in the reverse micellar system we have used the empirical polarity index $E_T(30)$ (developed by Dimroth and Reichardt) (22). For this purpose, the emission maximum (λ_{em}^{max}) of TOE was first determined in different homogeneous solvents for which $E_T(30)$ values are known (22). These data (Table 1) were used to draw a calibration plot (Fig. 3a) from which we estimated the empirical polarity parameter $E_T(30)$ in reverse micelles for different values of w_0. At $w_0 = 1$, the $E_T(30)$ values of TOE was found to be ~30.17. As shown in Fig. 3b, with increasing water content $E_T(30)$ increases rapidly until $w_0 = 15$, and relatively slowly thereafter. At the highest w_0 examined, i.e., $w_0 = 40$, the $E_T(30)$ value is 48.67 which is much lower that that of bulk water for which $E_T(30) = 63.1$. The intrinsically low quantum yield of TOE in water results in lack of perceptible spectral features in the steady state emission profile of TOE in this medium (spectrum not shown).

Figure 4 shows the fluorescence anisotropy (r) (λ_{em} = 340 nm) of TOE in AOT/*n*-heptane reverse micelles plotted against w_0. It is evident that as w_0 is increased, r gradually decreases. This observation can be attributed to the partitioning of more and more probe molecules from the head group region toward the bound water phase with increase in w_0. From w_0 ~ 13–15 onwards, further change in anisotropy (r) value gradually decreases, indicating that there is no appreciable additional change in the average mobility of TOE molecules. It is evident from Fig. 3b and Fig. 4 that even at very

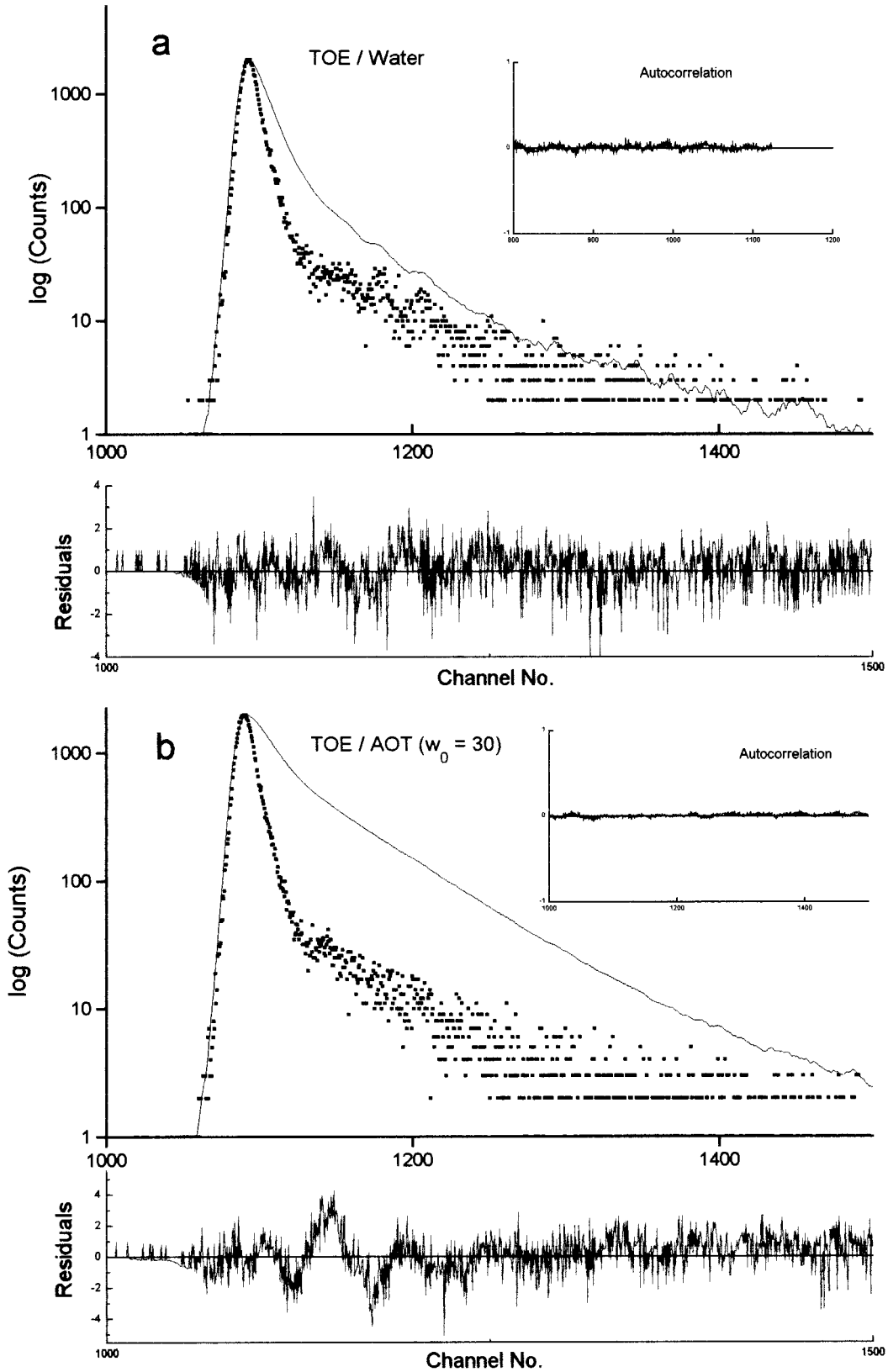


FIG. 5. Typical fluorescence decay curves and the results of a double exponential analysis for TOE (a) in aqueous solution and (b) in AOT/*n*-heptane reverse micelles at $w_0 = 30$. The timing calibration was 0.051 ns/channel. Recovered decay parameters are presented in Table 1 and Table 2. Emission was monitored at 297 nm. Solid line indicates the decay profiles. The lamp profiles are indicated by the unconnected points. The lower plots show the weighted residuals and the inset plots display the autocorrelation function of the weighted residuals.

high values of w_0, the TOE molecules do not reach the bulk water phase which prevails at the micellar core.

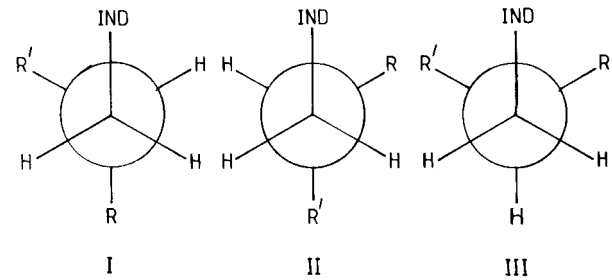
We also carried out preliminary lifetime measurements of TOE at representative values of w_0 as well as in different representative homogeneous solvents. The lifetime data in different environments are given in Fig. 5, Table 1 and Table 2. It is noteworthy that TOE exhibits double exponential fluorescence decay in all the solvents examined. This behaviour is reminiscent of tryptophan (which exhibits nonexponential decay in water) (16, 27, 28). Like tryptophan, the presence of three rotamers about the C_α—C_β bond (structure shown in Scheme 1) (16, 27, 28), gives TOE a structural heterogeneity, which would account for the nonexponential decay of TOE in homogeneous solutions.

As in the case of homogeneous solvents, double exponential fluorescence decay characteristics are also noted for TOE in AOT reverse micelles at all the w_0 examined (see Table 2). In this case the biexponential decay may be attributed to a combination of two possible factors, namely structural heterogeneity (as discussed earlier) as well as heterogeneity in the microenvironment of the fluorophore in the reverse micelles. From Table 2, it is evident that even at high water content in reverse micelles (e.g., $w_0 = 40$), the average lifetime ($\langle\tau\pm$) does not reach the limiting value corresponding to the bulk water phase (see Table 1). A comment is in order regarding the observation that with variation in w_0, $\langle\tau\pm$ undergoes only rather modest changes (when $w_0 = 1$, $\langle\tau\pm = 3.30$, and when $w_0 = 40$, $\langle\tau\pm = 3.51$) in contrast to the significant changes in λ_{em}^{max} value for a similar change in w_0. This may be attributed to the fact that unlike λ_{em}^{max}, which is largely determined by environmental polarity, the observed lifetime value depends on both radiative as well as nonradiative decay parameters, some of which are likely to depend on other factors besides polarity. The red shift in λ_{em}^{max}, along with decrease in the fluorescence anisotropy (r) with increasing w_0, are consistent with the picture that with increase in w_0 the TOE molecules come out of the head group region and enters the bound water phase. It may be noted that even at the highest w_o examined ($w_o = 40$) r is still higher ($\approx$0.04) than that obtained for TOE in bulk water ($\approx$0.01, shown by the dotted line in Fig. 4) which suggests that even at very high water content (e.g., $w_o = 40$), the TOE molecules do not locate entirely in the bulk water phase prevailing at the micellar core.

TABLE 2

Fluorescence Decay Parameter of TOE in AOT/*n*-Heptane Reverse Micelles at Various Values of w_0

w_0	τ_1 (nsec)	A_1	τ_2 (nsec)	A_2	$\langle\tau\rangle$ (nsec)
1	4.99	55.86	1.17	44.19	3.30
2	5.02	61.11	1.16	38.89	3.52
4	5.26	57.33	1.16	42.67	3.51
8	5.19	56.54	1.12	43.46	3.42
10	5.13	57.85	1.24	42.15	3.49
15	5.04	60.45	1.03	39.55	3.45
20	5.07	61.54	1.08	38.46	3.53
40	5.05	61.52	1.04	38.48	3.51



SCHEME 1. Structures of the three rotamers of TOE along the C_α—C_β bond in Newman projections. IND is the indole ring, R′ is the NH_2, and R is the COO—$(CH_2)_7$—CH_3 group. (Ref. 16, 27, 28).

In conclusion, the present research focuses attention on novel features of the fluorophore TOE, namely the significant sensitivity of its emission parameters (especially λ_{em}^{max}, relative emission intensity and fluorescence anisotropy) to the water content (i.e., w_0 values) of reverse micelles. These findings strongly suggest the potential usefulness of TOE as a probe for membrane proteins and peptides occurring in water restricted environments prevailing at the interfaces of biomembranes.

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