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Photophysical Study of Local Anesthetics in Reverse Micelles and Water-Ethanol Mixtures

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Fluorescence emission from three tertiary amine local anesthetics, dibucaine (D), tetracaine (T), and procaine (P) was investigated in the membrane-mimetic environment of AOT/isooctane reverse micelles and water-ethanol mixtures. At room temperature all three show broad UV absorption bands and large Stokes' shifts in emission from aqueous medium. In reverse micelles the emission bands are blue-shifted, with the magnitude of this shift decreasing continuously as the water-to-surfactant molar ratio (w_o) of the micelles is increased. The emission intensity of D is much stronger in water and in the interior of micelles than in ethanol, whereas for T and P the emission in water is much weaker than in ethanol and in micelles. Whereas the behavior in micelles is the result of the increased hydrophobicity with respect to bulk water in their interior, that in water-ethanol mixtures may be explained on the basis of state reversal of the excited electronic levels of D with increasing hydrogen bonding capability of the solvent mixture. The steady-state anisotropy for both T and P is unusually high in bulk water (~0.27) and increases with increasing w_o in micelles. However, the rotational correlation time of the anesthetics, as calculated using Perrin's equation, decreases with increasing w_o, as expected from physical considerations relating the size of the water pool to the ease of rotational relaxation of probe molecules inside micelles. Together, these results point to a marked difference in emission properties between D on one hand and T and P on the other, arising from differences in electronic characters of their primary emitting chromophores—quinoline for D, para amino benzoate for T and P.

KEY WORDS: Local anesthesia; reverse micelles; photophysics; fluorescence anisotropy; hydrophobicity.

INTRODUCTION

Tertiary amine local anesthetics cause anesthesia by blocking nerve transmissions through state-dependent binding of voltage-gated Na^+-channels [1,2]. It is generally believed that interaction of the anesthetic molecules with membrane lipids or membrane proteins leads to the inactivation of neuronal ion channel activity. However, of the two the first has to be an important first step for local anesthesia, because transbilayer movement of anesthetics to the cytoplasmic side of excitable membranes is a requirement for anesthetic action [2,3]. Considerable effort has thus been spent on studies of the behavior of local anesthetics in presence of membranes and membrane-like environments, as well as on the effect of anesthetics on the organization and dynamics of membrane lipids [4–13].

Dibucaine (2-butoxy-*N*-[2-diethylaminoethyl]-4-quinoline-carboxiamine), tetracaine (2-dimethylamino-ethyl-4[*n*-butylamino]benzoate), and procaine (2-diethylamino-ethyl-4-aminobenzoate) are some of the most widely studied tertiary amine local anesthetics (Fig. 1). Depending on the pH of the solvent and the nature of the microenvironment,

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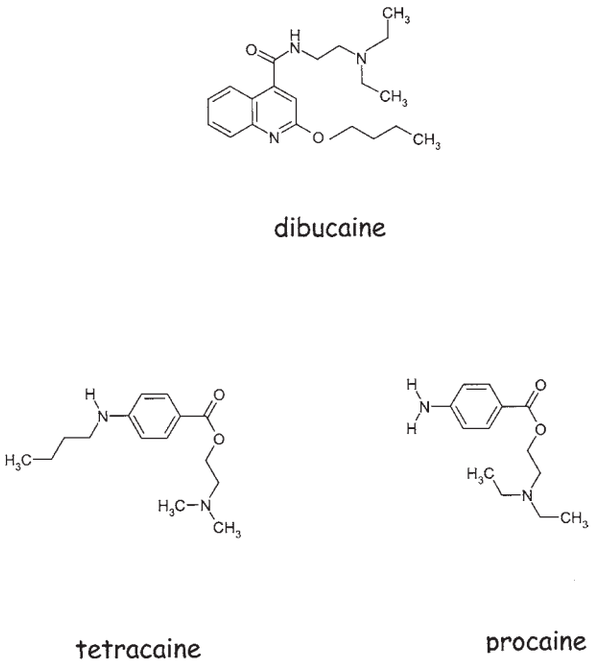

Fig. 1. Structures of the three anesthetic molecules.

each can exist in neutral, in singly or doubly positively charged form, or as aggregated species [14,15]. Fluorescence and NMR studies have shown them to penetrate into phospholipid bilayer vesicles, indicating the amphipathic nature of these molecules [5–8,10,11]. The effect on the membrane location of the anesthetics caused by the presence of cholesterol, a ubiquitous component of the plasma membrane, in the bilayers also has been investigated, leading to the conclusion that cholesterol "squeezes out" the anesthetics from the hydrophobic regions of the bilayers [16,17].

Here we report a comparative study of these three local anesthetic molecules in the membrane-mimetic environment existing inside reverse micelles of AOT (sodium *bis*(2-ethylhexyl) sulfosuccinate)/water/isooctane. Reverse micelles are closed aggregates of surfactants (AOT) in hydrocarbon solvents (isooctane) having the polar head groups of AOT lining the inner surface of the aggregated structures while the alkane tails protrude into the isooctane phase [18,19]. The aggregates are monodisperse and can entrap a limited amount of water, the amount of which (characterized by the water-to-surfactant molar ratio, $w_o = [H_2O]/[AOT]$) controls the size and aggregation number of the micelles, as well as the physicochemical properties of the entrapped water [20–22]. For small w_o (≤ 10) the solubilized water has properties (e.g., polarity, microviscosity, dielectric relaxation time) similar to those of interfacial water present near biologic membranes or protein surfaces. With addition of more water, that is, increasing w_o, a water pool with bulk-like properties begin to form within the micelles. Thus reverse micelles offer a very convenient way of following the spectroscopic properties of molecules dissolved in the water pool as the character of the solvent water changes from the highly structured, interfacial type to the more relaxed, bulk type with increasing water content, that is, with increasing w_o [23]. Being optically transparent, solutions of reverse micelles lend themselves readily to spectroscopic investigations of entrapped species using techniques such as absorption and fluorescence.

Mixtures of water and alcohol constitute another interesting series of solvents whose dielectric constant and hydrogen-bonding capability vary with the relative fractions of the individual components and mimic the conditions occurring in varied biologic media. Photophysical studies on the anesthetics were also performed in water-ethanol mixtures and the results compared with those obtained in reverse micelles. The results of our investigations suggest that there is a qualitative difference in the molecular properties of the local anesthetics in solution, despite their apparent similarity of structure and function.

EXPERIMENTAL

Materials and Methods

High-purity dibucaine · HCl, tetracaine · HCl, procaine · HCl, and AOT were purchased from Sigma (St. Louis, MD, USA). Isooctane and ethyl alcohol (Uvasol grade) were from E. Merck (Darmstadt, Germany). Triple distilled water was used for preparing solutions and buffers.

Concentrated stock solutions of the anesthetics were prepared in ethanol. To incorporate them in reverse micelles of a given w_o, the requisite volume (a few μL) of each was injected into 1—2 ml of a 50-m*M* solution of AOT in isooctane and shaken till clear. During each set of measurements w_o was increased stepwise from 2 to 60 by adding small volumes of water to the same AOT-isooctane solution.

The concentrations of the anesthetics were determined spectrophotometrically, using extinction coefficients of 4400 M^{-1}/cm^{-1} at 326 nm for dibucaine, 23,000 M^{-1}/cm^{-1} at 311 nm for tetracaine and 12,000 M^{-1}/cm^{-1} at 290 nm for procaine [8,24]. Absorption spectra were measured on a Hitachi UV-3300 spectrophotometer. Corrected fluorescence spectra were recorded on a Hitachi F-4010 spectrofluorometer, using excitation at 326, 311, and 290 nm for dibucaine, tetracaine, and procaine,

respectively. A bandpass of 3 nm was used in both excitation and emission channels, and appropriate baseline subtractions were made. Optical densities of fluorescent samples at their excitation wavelengths were kept below 0.1. Quantum yields were determined, using similar instrument parameters, on a Jobin-Yvon Horiba Fluoromax-3 spectrofluorometer relative to that (ϕ_{trp}) of a solution of L-tryptophan in phosphate buffer, pH 7.0, with ϕ_{trp} being taken to be 0.14 [25]. Steady-state anisotropy was also measured on this instrument using L-format optics and slits of bandwidth 5/10 nm in excitation/emission. All measurements were performed at 25°C with multiple (3–5) sets of samples. The limiting anisotropy (r_o) of each sample, defined as its anisotropy in a rigid medium at very low temperature, was determined by extrapolating to zero abcissa the *r versus* T/η graph, obtained by measuring its anisotropy in glycerol in the temperature range 10°C–40°C.

Fluorescence lifetimes of dibucaine were determined from total emission intensity decay measurements using a nanosecond time-domain fluorometer assembled in our laboratory and operated in the time-correlated single-photon-counting mode, using excitation at 337 nm and measuring the decays at the wavelength of emission maximum in each solvent [17]. Slits of bandwidth 16 nm were used in both excitation and emission channels. Intensity decay curves were fitted to the sum-of-exponentials series

$$I(t) = \Sigma_i A_i \exp(-t/\tau_i)$$

where A_i is a factor representing the fractional contribution to the time-resolved decay of the component with a lifetime of τ_i. Decay parameters were estimated using a software package supplied by Edinburgh Instruments, Inc. (UK), implementing a nonlinear least-squares iterative fitting procedure. Mean lifetimes were calculated using the relationship

$$<\tau> = \Sigma_i A_i\tau_i^2 \cdot / \Sigma_i A_i\tau_i$$

Fluorescence lifetimes of tetracaine and procaine were measured in a picosecond time-resolved fluorescence spectrometer where the excitation was provided by "vertically" polarized laser pulses (pulse width ~2 ps) of wavelength 307 nm from a high-repetition rate (4 MHz) Tsunami laser. Fluorescence emission at magic angle (54.7 degrees) was dispersed in a monochromator (*f*/4, spectral width 2.5 nm) and counted ($4–5 \times 10^3/s^{-1}$) by a micro-channel plate photomultiplier (R2809). Fluorescence decays, collected at 20 ps/channel resolution, were measured at the wavelengths of respective emission maxima in various solvents. The instrument response function was ~40 ps (see Ref. 26 for further details).

RESULTS

Absorption spectra of all three anesthetics in water showed single broad peaks in the UV region, at 326 nm, 312 nm, and 290 nm for dibucaine, tetracaine, and procaine, respectively (Table I). Fluorescence emission from each in aqueous medium also exhibited a single maximum, at the wavelengths (λ_{max}) 412 nm, 372 nm, and 355 nm for dibucaine, tetracaine, and procaine, respectively (Fig. 2).

In reverse micelles of AOT/water/isooctane, the positions of the absorption peaks remained practically unchanged for all three molecules and over the whole range of w_os (2–60) investigated. However, large blue shifts of the emission spectra were observed upon their incorporation in the micelles (Fig. 2). The magnitude of this blue shift was the largest at the lowest w_o and decreased with increase in water content of the micelles, that is, with w_o (Fig. 3). The increase of λ_{max} with w_o largely leveled off beyond w_o ~15. For all three samples, however, the saturation value of λ_{max} for emission from reverse micelles was considerably smaller than that for emission from bulk water. The highest values of λ_{max} observed in micelles (at $w_o \geq 50$) were 396 nm, 353 nm, and 342 nm for dibucaine, tetracaine, and procaine,

Table I. Spectroscopic Properties of Local Anesthetics in Different Solvents Concentrations Used : [D] = 10 μ*M*, [T] = 4 μ*M*, [P] = 2 μ*M*.

Solvent	Absorption Maxima (nm)			Steady-State Fluorescence Emission Parameters								
				λ_{max} (nm)			Quantum Yield (Φ)			Anisotropy		
	D	**T**	**P**	**D**	**T**	**P**	**D**	**T**	**P**	**D**	**T**	**P**
Ethyl alcohol	327	309	295	388	358	345	0.02	0.14	0.05	0.05	0.073	0.139
Water	326	312	290	413	372	356	0.17	0.003	0.002	0.007	0.263	0.264

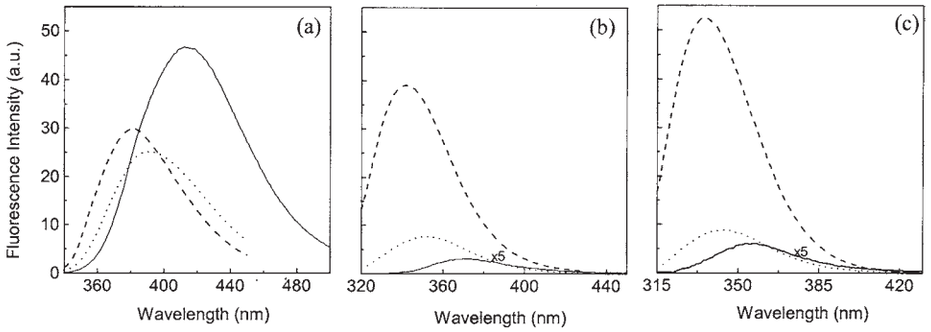

Fig. 2. Fluorescence emission spectra of (a) dibucaine, (b) tetracaine, and (c) procaine in water (—) and in reverse micelles at $w_o = 2$ (---) and $w_o = 50$ (. . . .). The spectra of tetracaine and procaine in water were enlarged 5 times to make them visible on this scale.

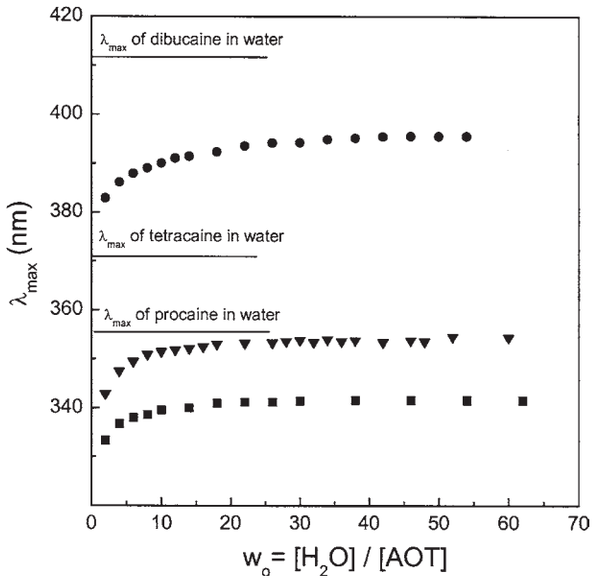

Fig. 3. Variation of wavelength at emission maximum (λ_{max}) of dibucaine (●), tetracaine (▼), and procaine (■) with water content (w_o) of reverse micelles. The value of λ_{max} for each fluorophore in bulk water is also indicated by a solid line.

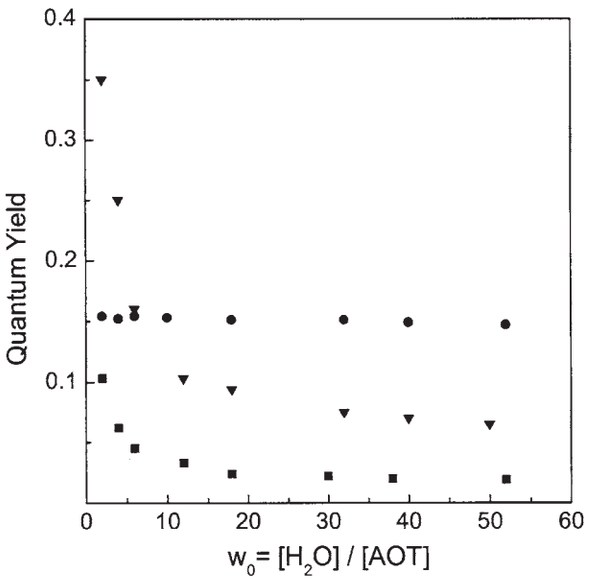

Fig. 4. Quantum yield of dibucaine (●), tetracaine (▼), and procaine (■) versus w_o in AOT/water/isooctane reverse micelles.

respectively, these being about 15 nm smaller than the corresponding λ_{max} observed in water (Fig. 3).

Quantum yields of the anesthetics in aqueous medium showed large variations, from 0.18 for dibucaine to 0.003/0.002 for tetracaine/procaine (Table I). The quantum yield of dibucaine was somewhat lower in reverse micelles than in bulk water and remained almost constant at the value 0.15 for all w_o (Fig. 4). On the contrary, for tetracaine and procaine the yields in micelles were about 50–100 times larger than in water (0.35 and 0.11, respectively, at $w_o = 2$) and decreased very rapidly with increase in w_o (0.07 and 0.02, respectively, at $w_o = 50$, Fig. 4). When normalized to the same value at $w_o = 2$, the yield *versus* w_o curves for these two molecules were identical (not shown).

Figure 5 shows the variation of steady-state anisotropy of the anesthetics with the water content of reverse micelles. The anisotropy of dibucaine was very small (0.007) in bulk water but considerably higher in micelles for all w_os; it was 0.065 at $w_o = 2$ and decreased slowly to 0.05 at $w_o = 55$. On the contrary, the anisotropy values of both tetracaine and procaine were unusually high (0.26) in bulk water (Table I). However, the most surprising observation was that for both these molecules the anisotropy in reverse micelles was lower than in water and an increasing function of w_o: it was 0.16 at $w_o = 2$, increased to 0.22 at $w_o = 15$, and remained at this value up to $w_o = 55$ (Fig. 5).

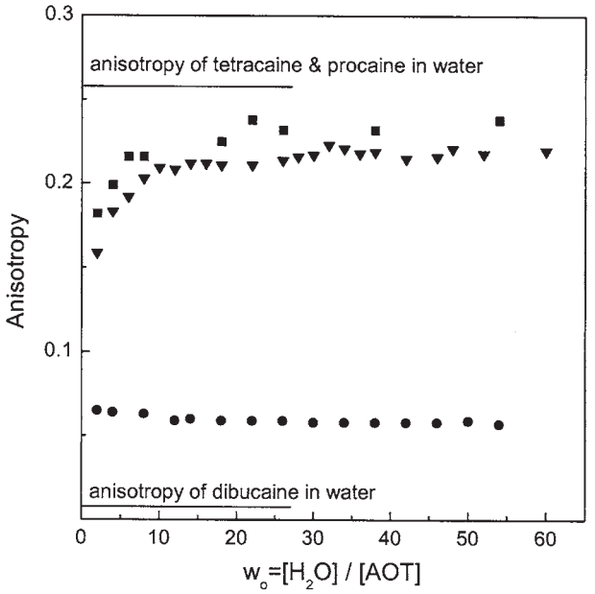

Fig. 5. Steady-state fluorescence anisotropy of dibucaine (●), tetracaine (▼), and procaine (■) versus w_o in reverse micelles. The anisotropy values of the fluorophores in bulk water are also indicated in the figure.

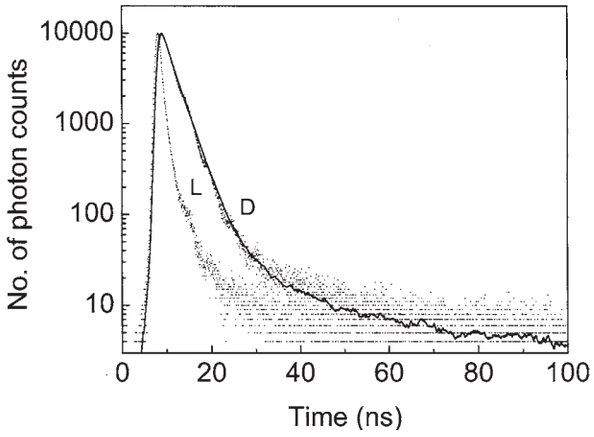

Fig. 6. Total emission intensity decay profile of dibucaine in reverse micelles at $w_o = 2$. The dotted curve marked "L" shows the lamp profile, while the solid line marked "D" represents fit of the data to a sum of two exponential decays (see text).

Analysis of time-resolved fluorescence emission from all three molecules yielded mostly biexponential decays, both in bulk solvents and in reverse micelles. Figure 6 shows a representative decay trace and the quality of the fit for dibucaine in reverse micelles at $w_o = 2$. For dibucaine in water, the two lifetime components were ~3 ns and ~1 ns, with respective contributions of ~80% and ~20%. Both components were reduced in micelles, to about 2.6 ns and 0.8 ns but with the same relative contributions, with w_o varying from 2 to 50. The mean lifetime was thus reduced from 2.7 ns in water to 2.1–2.3 ns in micelles (Table II). For both tetracaine and procaine the lifetime in bulk water was too short (<0.02 ns) to be measured even with the picosecond lifetime spectrometer setup. In reverse micelles, the lifetime was longer at low w_o (~1 ns for $w_o = 2$) and decreased with increasing w_o (Table II).

In going from water to ethanol, the wavelength of the absorption maximum remained unaltered for dibucaine but exhibited a blue shift of 3 nm for tetracaine and a red shift of 5 nm for procaine (Table I). Fluorescence maxima were blue-shifted with respect to emission from bulk water, by amounts ranging from 10 nm (procaine) to 25 nm (dibucaine). The quantum yield decreased by almost an order of magnitude for dibucaine, while increasing even more for tetracaine and procaine (25–50 times). Steady-state anisotropy values showed the opposite trend, increasing substantially for dibucaine while decreasing for tetracinae/procaine. Figure 7 illustrates the continuous variation of quantum yield and anisotropy of all three anesthetics with the volume fraction of ethyl alcohol in water-alcohol mixtures. For tetracaine and procaine the yield increased and the anisotropy decreased smoothly with increasing alcohol content, whereas for dibucaine just the opposite behavior was observed. For dibucaine, the individual lifetime components in ethanol were about an order of magnitude smaller than in water, leading to a considerably shorter mean lifetime (Table II). For both tetracaine and procaine the mean lifetime in ethanol was at least an order of magnitude longer than in water.

DISCUSSION

The optical properties of the tertiary amine local anesthetics are determined by the quinoline analogue in dibucaine and the benzene analogue in tetracaine and procaine (Fig. 1) (see Refs. 14,15). Depending on the solution conditions, each of these can exist in the neutral, hydrogen-bonded, mono- or dicationic forms. In water, at the pH (~6.5) at which these measurements were carried out, all three are likely to be in their monocationic forms, whereas in ethanol they would all be deprotonated, that is, neutral [15].

The wavelengths of the absorption maxima of the anesthetics in water and ethanol (Table I) agree well with those observed earlier by Vanderkooi *et al.,* indicating that the molecules exist in the monocationic and deprotonated

Table II. Fluorescence Lifetimes of Local Anesthetics in Different Solvents (in nanoseconds)

Medium	Dibucaine		Tetracaine		Procaine	
	$<\tau>$	$<\tau_c>$	$<\tau>$	$<\tau_c>$	$<\tau>$	$<\tau_c>$
RM ($w_o = 2$)	2.1	0.76	1.25	1.55	0.86	1.76
RM ($w_o = 8$)	2.2	0.77	0.41	1.00	0.11	0.43
RM ($w_o = 20$)	2.2	0.70	0.27	0.76	<0.02	0.11*
RM ($w_o = 50$)	2.3	0.73	0.21	0.70	<0.02	0.12*
Bulk water	2.7	0.08	<0.02	0.23*	<0.02	0.75*
Ethanol	0.44	0.11	0.35	0.12	0.3	0.11

Excitation was at 337 nm for dibucaine and at 307 nm for tetracaine and procaine. Emission was monitored at respective emission peaks. Mean total intensity decay lifetimes ($<\tau>$) and mean rotational correlation times ($<\tau_c>$) were calculated (using Perrin's equation) as described in the text. Values marked with asterisks indicate upper limits of $<\tau_c>$ based on upper limits of corresponding values of $<\tau>$.

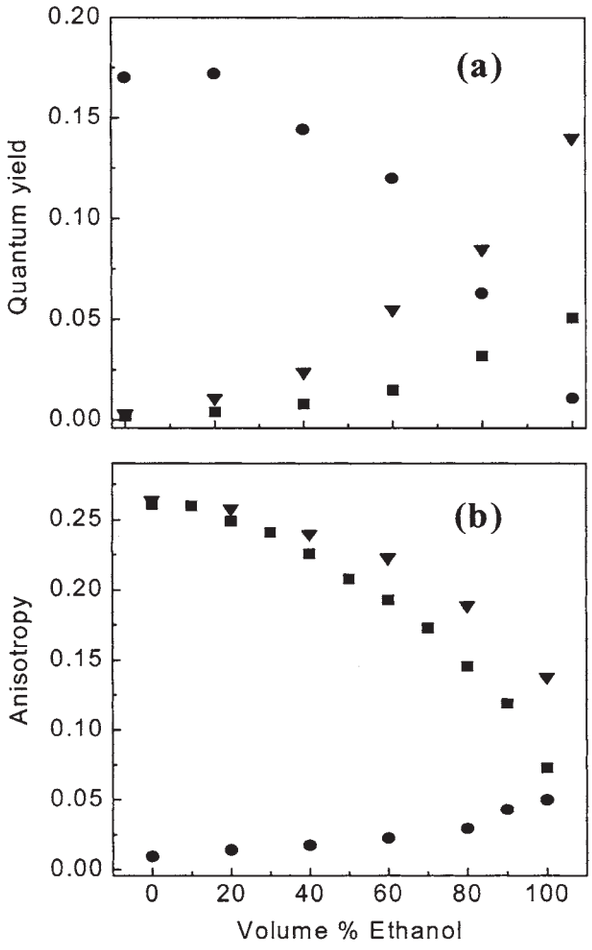

Fig. 7. (a) Quantum yield and (b) steady-state anisotropy versus volume percent of ethyl alchohol in water-alchohol mixtures for dibucaine (●), tetracaine (▼), and procaine (■).

states in water and ethanol, respectively [14,15]. In reverse micelles the positions of the absorption peaks correspond to those of the neutral species of the molecules. Both dibucaine · HCl and tetracaine · HCl are very sparingly soluble in isooctane and do not show any characteristic absorption and fluorescence spectra in it. Partitioning of local anesthetics in the organic phase is thus not considered in explaining the fluorescence results.

The blue shifts of the fluorescence maxima, observed upon incorporation of the anesthetics in reverse micelles, point to the fact that the micellar milieu constitutes a hydrophobic medium with effective dielectric constant lower than that of bulk water [23]. The blue shifts expectedly decreased with increasing w_o, because the addition of more water creates a bulk-like water pool (of increasing dielectric constant) within the micelles. However, the result that even at the highest w_o the emission maxima in micelles were considerably blue-shifted (~15 nm) with respect to those found in bulk water implies that all three probes are preferentially localized in the interfacial region of the micelles, which retains its hydrophobic character even in presence of an increasing water pool.

Acquisition of bulk-like properties by the water pool also accounts for the decrease of quantum yield with increase in w_o (Fig. 4), because the presence of such water makes more nonradiative pathways available to the excited anesthetic molecules. The pattern of fluorescence lifetimes ($<\tau>$) in micelles (Table II) is consistent with the behavior of the quantum yield; for dibucaine, both parameters show little change on varying w_o, whereas for tetracaine/procaine they decrease by factors of 5–10 as w_o increases from 2 to 54. The highly structured water molecules of the micellar pool have been shown to constitute a rigid medium allowing restricted rotational mobility of solvated molecules. This explains why the

fluorescence anisotropy of dibucaine is much higher in micelles than in water, as well as the small decrease in its anisotropy at higher w_o as the water environment becomes more relaxed (Fig. 5).

An important aspect of the results reported here is the marked difference in fluorescence behavior between dibucaine on one hand and tetracaine and procaine on the other, in both bulk solvents and reverse micelles. For example, the quantum yield of dibucaine in micelles is 40–70% lower than in water, whereas that of tetracaine/procaine is 30–100 fold higher. This fact is also reflected in the ratio of the mean fluorescence lifetimes in micelles and in water (Table II). Again, whereas the anisotropy of dibucaine is expectedly higher in the interior of micelles than in bulk water, that of tetracaine/procaine is lower in micelles than in bulk water and increases with increasing w_o. To our knowledge, this is the first observation of fluorescence anisotropy being lower in a rigid, structured medium (micelles) than in a bulk solvent, where the solvent molecules are free to move about. The result that the anisotropy of tetracaine/procaine actually increases with w_o is also contrary to what one would expect from conventional arguments; as more water is injected into the central pool, a relatively mobile network of hydrogen-bonded water molecules is formed, freeing up the solvated anesthetics in the process. This should lead to enhanced rotational freedom of the fluorophore within the micelle, and hence decrease its anisotropy.

The difference in fluorescence yields between dibucaine and the other two anesthetics can be explained by considering the nature of their emitting (electronic) states and the effect of the solvent medium on them. Fluorescence emission from the neutral species of dibucaine, whose emitting chromophore has been shown to be the quinoline analogue (Fig. 1), occurs from the lowest lying singlet n, π^* excited state. In protic solvents such as ethanol, interactions leading to the formation of hydrogen bonds with the nitrogen lone pair of quinoline can cause the relative positions of the n, π^* and π, π^* singlet states to be interchanged, with the latter now becoming the lowest-lying excited state. When the hydrogen bonding interaction becomes stronger (as in water), the triplet n, π^* state will be raised still higher above the singlet π, π^* state [27]. This will effectively reduce radiationless transitions (intersystem crossing) from the latter states to the former and bring about an increase in the fluorescence yield. The continuous decrease in quantum yield of dibucaine in water-ethanol mixtures, varying from pure water to pure ethanol (Fig. 7[a]), can be explained on the basis of the above argument. No such state reversal is expected to occur for tetracaine and procaine, for both of which the emitting chromophore is the benzene ring (Fig. 1), and emission occurs from the lowest π, π^* singlet states. The increase in quantum yield observed for these molecules in going from water to ethanol (Fig. 7[a]) is thus attributed to the decrease in solvent polarity, the factor also responsible for enhancing the yield at low w_o in reverse micelles (Fig. 4).

An alternative explanation for the reduction of quantum yield in ethanol compared to water can be formulated based on the work of Coutinho *et al.* [8]. The effect is attributed to an intramolecular charge transfer quenching operative in neutral dibucaine (the ethanolic species), whose terminal aliphatic amine acts as the electron donor and the quinoline moiety as the acceptor. Upon protonation of the amino group (as in water) its ionization potential increases, rendering electron donation unfavorable. Evidence for the existence of such a mechanism is provided by pH-dependent studies of quantum yield (Φ_D) and lifetime (τ_D) of dibucaine in water [28]. There it was found that for the charged species $\Phi_D^+ = 0.25$, $\tau_D^+ = 3.33$ ns, whereas for the neutral species $\Phi_D = 0.033$ and $\tau_D = 0.77$ ns, in good agreement with the values obtained in the present work for these parameters in water and ethanol, respectively (Tables I and II). This mechanism is not operative for the other anesthetics because the reduction potential of the benzene derivatives is not sufficiently favorable.

The implications of anisotropy data may be understood by calculating the rotational correlation time (τ_c) of each fluorophore in various solvents using Perrin's equation [29]:

$$\tau_c = <\tau>/(r_0/r - 1)$$

where r_0 is the limiting anisotropy for the fluorophore, determined by extrapolation of the *r-vs.*-T/η graph as described in the Materials and Methods section. The values of r_0 so determined for dibucaine, tetracaine, and procaine were 0.24, 0.29, and 0.27, respectively. Values of τ_c calculated using these r_0-values are listed in Table I. In bulk water or ethanol, τ_c is very small (~0.1 ns), whereas it is about an order of magnitude larger in micelles. These results are consistent with estimates of τ_c obtained from studies of other fluorophores in AOT/isooctane reverse micelles [30]. Table II also shows that for all three samples τ_c decreases with increasing w_o. Because τ_c is a measure of the resistance of the solvent medium to its rotational motion, this implies that the ease of rotational diffusion of the fluorophores indeed increases as the size of the micellar water pool increases, as expected from the arguments given above. For tetracaine and procaine, the reason for the observed increase of anisotropy with increasing w_o can thus be attributed to a faster rate of decrease of

the fluorescence lifetime ($<\tau>$) over that of the correlation time (τ_c). This in turn implies that interactions with surrounding water molecules are much more effective in creating new deactivation pathways for their excited states, as evidenced by the sharp fall in their quantum yields with increasing w_o in micelles (Fig. 4), than in reducing the local microviscosity of the solvent shell around them.

The results reported here show that the photophysical behavior of the local anesthetic, dibucaine is distinctly different from that of the other two, tetracaine and procaine. This can be attributed to the difference in electronic character, specifically, energy level structures, of their emitting chromophores, namely quinoline for dibucaine and para amino benzoate for tetracaine and procaine. This difference may lead to different interactions of the anesthetics with membrane lipids and proteins involved in producing local anesthesia and may play a role in their levels of efficacy and toxicity.

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