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Biochimica et Biophysica Acta 1190 (1994) 319–328

BBA Biochimica et Biophysica Acta

Charge- and pH-dependent binding sites for dibucaine in ionic micelles: a fluorescence study

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(Received 1 July 1993; revised manuscript received 15 November 1993)

Abstract

Binding of micromolar concentrations of the local anesthetic dibucaine to micelles of cationic, zwitterionic and anionic detergents was studied using the fluorescence emission of dibucaine. Difference in quantum yields for charged and neutral dibucaine allowed to obtain shifts of pK_a values due to binding. Estimates for the electrostatic potential affecting the tertiary amine of dibucaine were obtained from the pK_a shifts. Change of fluorescence emission upon binding allowed to obtain the binding constants of both charged and neutral dibucaine to the micelles. The binding constant for the neutral form is essentially independent of micelle charge and of specific differences in detergent structure. Consistency between the ratio of neutral to cationic dibucaine binding constants and the measured pK_a shift was tested. For LPC micelles complete agreement was found. For CTAC, however, the ratio of binding constants does not explain the pK_a shift. The discrepancy between the results is used to estimate the errors involved upon neglecting non-coulombic electrostatic interactions of drugs to charged membrane surfaces. Fluorescence quenching with sodium iodide and nitroxide stearic acid derivatives allowed a depth profiling of the drug in the micelles.

Key words: Micelle; Dibucaine; Local anesthetic; Fluorescence spectroscopy

1. Introduction

The chemical structure of many local anesthetics involves a tertiary amine and aromatic rings. The tertiary amines have a pK near the physiological pH [1–3] and, consequently, occur in both charged and uncharged forms upon interacting with various biological systems. The aromatic rings are responsible for rich fluorescence properties, which varies with the protonation state and the local environment of the drug [4–6], and thus can be used for probing the anesthetic interactions.

Many biological processes occur at the surface of membranes or within their hydrophobic moiety. Owing to ionizable headgroups of lipids, the surface of biological membranes frequently presents a net charge giving rise to different binding properties of charged and uncharged forms of molecules possessing ionizable groups. A lot of work has been done on the tertiary amine local anesthetics concerning functional effects on biological systems and structural and physico-chemical properties in several environments. In addition to the effect of blocking nerve transmission they promote a variety of other changes on biological systems [7–10]. The effect of local anesthetics on the organization of lipid bilayers and the decrease in the freedom of motion of anesthetic molecules upon binding to lipid membranes were studied by several techniques [1,11,12]. Multiple binding sites for tetracaine and procaine in phospholipid bilayers were observed using deuterated anesthetics [2]. Despite the large amount of work, the detailed mechanisms of action of these drugs at the molecular level remains unclear. For this reason more information is necessary from model systems.

Micelles have been used to mimic biological membrane environments [13–16] and provide simple sys-

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SSDI 0005-2736(93)E0344-A

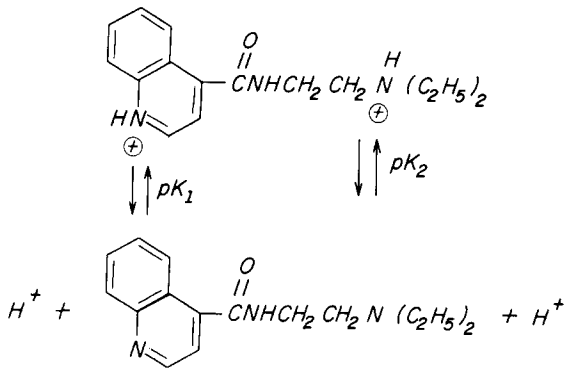

Fig. 1. Equilibrium of the ionization states of dibucaine.

tems to study the physico-chemical properties of binding of amphipathic molecules to membranes. To properly analize data on the interactions of ionizable drugs with membrane systems it is important to know the actual concentrations of both the charged and uncharged forms of the drug at the membrane. This can be achieved by knowing the binding constants of each form of the drug to the system under consideration and the shift on the apparent pK of the bound drug. The effect of neutral and charged micelles on the acid-base dissociation of tetracaine has been studied using tetracaine fluorescence [4], but no similar work has been done for dibucaine.

The absorption and fluorescence properties of dibucaine in aqueous solutions has been reported as a function of pH [5]. Dibucaine can exists in three states of ionization depending on the pH of the medium (Fig. 1). The pK for the aromatic N (pK_1) is very low (1.77), whereas the pK for the tertiary amine (pK_2) lies in the alkaline range (8.95) [5]. Low temperature (77 K) fluorescence and phosphorescence of dibucaine solubilized in different micelles provided an attempt to get some insights on the micellar solubilization sites [6]. Unfortunately this work did not properly take into account the amount of different anesthetic species.

In the present work we used the fluorescence emission of dibucaine to study its acid-base dissociation in charged (CTAC and SDS) and zwitterionic (LPC) micelles in order to determine the shifts on the apparent pK of micelle-bound anesthetic caused by the micelle surface charges. We also used the changes of the emission spectrum to find the binding constants of the charged and uncharged forms of dibucaine to these three micellar systems. Finally, experiments on quenching of dibucaine fluorescence by the ionic quencher iodide and by the spin labeled stearic acids with the nitroxide groups at the fourth and twelfth carbon atom of the chain were performed to further characterize the solubilization sites of each species.

2. Materials and methods

Dibucaine hydrochloride and lysolecithin (LPC) were purchased from Sigma, sodium dodecylsulphate (SDS) from Bio-Rad, and cetyltrimethylammonium chloride (CTAC) was obtained from Herga and recrystallized twice from methanol/acetone (1:4, v/v). Spin labeled stearic acids with the nitroxide groups at the fourth and twelveth carbon atom of the chain (4-SASL and 12-SASL) were kindly supplied by A.Watts (Biochemistry Department, University of Oxford). All other reagents were analytical grade.

Dibucaine solutions ($2 \cdot 10^{-5}$ M) were prepared in 0.067 M borate/citrate/phosphate (BCP) buffer. The buffers were prepared from a stock solution of 100 ml containing 34.4 ml of NaOH 1 N, volumes of H_3PO_4 1 N and of citric acid 1 N standardized to 10 ml of the 1 N NaOH solution, and 354 g of boric acid. The pH of the buffer was adjusted by adding aliquots of 1 M HCl solution to diluted stock solution. The detergents were added from concentrated aqueous solutions (0.80 M SDS, 0.68 M CTAC, and 0.10 M LPC).

The pH values were obtained with a Corning 130 pH meter equipped with a glass Ag/AgCl semimicro combination electrode. Absorption measurements were performed on a Shimadzu UV-180 spectrophotometer. Fluorescence measurements were performed on a Jasco FP-777 spectrofluorometer. Fluorescence was excited at 328 nm (maximum of the lowest energy absorption band of dibucaine) and the whole emission band was detected (340 to 550 nm, in most cases). 5 parameters were obtained from the emission spectra, e.g., the integrated intensity of the whole emission band ($A = \int F(\lambda)\, d\lambda$), the maximum intensity (F_{max}) and the corresponding wavelength (λ_{max}), and the intensities at 382 and 435 nm (F_{382} and F_{435}). The wavelengths for the two later parameters were chosen after performing subtractions of the emission spectra, normalized to the same integrated intensity, at different pH values and different micelle concentrations. The subtractions presented extremes around these two wavelengths. Fluorescence quantum yields were obtained using fluoresceine in 0.1 N NaOH as a standard (quantum yield = 0.92) and comparing the integrated intensity of the emission bands and absorbances measured under the same conditions [17].

Quenching experiments were performed using concentrated solutions of quenchers, sodium iodide (1 M), 4-SASL (14 mM or 100 mM in ethanol), and 12-SASL (24 mM and 160 mM in ethanol), to titrate solutions of dibucaine in buffer or in micelles of appropriate detergent concentrations. Small aliquots of the solutions of quenchers were added to the samples using Hamilton microsyringes. The quenching data were corrected for traces of fluorescent impurities in the solutions of SASLs.

Calculations of pK values and quantum yields

The equation for analyzing pH titration curves of a single ionization equilibrium is

$$\mathrm{pH} = \mathrm{p}K + \log(\alpha/(1-\alpha)) \quad (1)$$

where α and $(1-\alpha)$ are the mole fractions of the neutral and protonated forms, respectively. Then,

$$\alpha = [\mathrm{D}^0]/([\mathrm{D}^0] + [\mathrm{D}^+]) \quad (2)$$

where $[\mathrm{D}^0]$ and $[\mathrm{D}^+]$ are the molar concentrations of the neutral and protonated forms, respectively.

The fluorescence emission spectrum of a probe undergoing an acid-base dissociation equilibrium can be used to analyze this process. The integrated intensity of the whole emission band (A) is the sum of the integrated intensities of the individual components, e.g., the deprotonated and protonated species. Therefore

$$A = \alpha A^0 + (1-\alpha)A^+ \quad (3)$$

where A^0 and A^+ are the integrated intensities when only the deprotonated and the protonated species are present, respectively. A^0 and A^+ are proportional to the respective quantum yields q^0 and q^+ of the two species, and the proportionality constant, ξ was determined using fluoresceine as a standard.

$$A = \xi[\alpha q^0 + (1-\alpha)q^+] \quad (4)$$

The apparent quantum yield is $q = A/\xi$. Then:

$$q = \alpha q^0 + (1-\alpha)q^+ \quad (5)$$

Solving for α and replacing in Eq. (1) leads to:

$$\mathrm{pH} = \mathrm{p}K + \log((q-q^+)/(q^0-q)) \quad (6)$$

Expressing q as a function of the pH

$$q = (q^0 \cdot 10^{\mathrm{pH}} + q^+ \cdot 10^{\mathrm{p}K})/(10^{\mathrm{pH}} + 10^{\mathrm{p}K}) \quad (7)$$

This equation was used to fit the data of pH titrations of dibucaine in aqueous and micellar solutions.

Assessment of the binding constants of dibucaine to micelles

Considering the micellar solution as a two-phase system, the binding constant of a probe to the micelle can be defined as

$$K_b = [\mathrm{P_m}]/[\mathrm{M}][\mathrm{P_a}] \quad (8)$$

where $[\mathrm{P_m}]$ and $[\mathrm{P_a}]$ are the concentrations of the micelle-bound and the aqueous phase probe and [M] is the concentration of detergent.

The binding of a probe to membrane systems can be monitored by any spectroscopic parameter of the probe which changes when it enters the membrane from the aqueous phase [18]. If fluorescence is measured a parameter which can be considered is $\varepsilon = F/F_0$, where F_0 and F are the fluorescence intensities measured before and after detergent addition. The corresponding ratio of the integrated fluorescence intensities A_0 and A can also be considered. The titration method based on Basford and Smith [19], in which the detergent concentration is varied at constant probe concentration was chosen to find the binding constants of dibucaine to micelles. Standard equilibrium considerations show that the dependence of the parameter ε on the amount of detergent added to the solution which contains the probe is [18]:

$$\varepsilon - 1 = (\varepsilon_b - 1) - (\varepsilon - 1)/K_b \cdot [\mathrm{M}] \quad (9)$$

where ε_b is the fluorescence parameter when [M] tends to infinity.

A plot of $(\varepsilon - 1)$ vs. $(\varepsilon - 1)/[\mathrm{M}]$ gives a straight line with a slope of $1/K_b$. The binding constant K_b can therefore be obtained from this plot.

Another spectroscopic parameter, less sensitive to fluctuations of the spectrofluorometer and scattering by small bubbles in micelle solutions is the fluorescence intensity normalized to the total integrated area of the band. Two equations are obtained using F/F_0 and A/A_0 in Eq. (9), which lead to

$$\varepsilon' - 1 = (\varepsilon'_b - 1) - (A_0/A_b \cdot K_b)(\varepsilon' - 1)/[\mathrm{M}] \quad (10)$$

where $\varepsilon' = F \cdot A_0/F_0 \cdot A$ and A_b is the integrated fluorescence when [M] is very large. To find K_b from the plots of $(\varepsilon' - 1)$ vs. $(\varepsilon' - 1)/[\mathrm{M}]$ it is necessary to correct the slope of the curves with the factor A_b/A_0, which is available when the quantum yields of aqueous and micelle-bound probes are known.

3. Results and discussion

Effect of micelles on the apparent pK of dibucaine

Dibucaine fluorescence was used by Vanderkooi to study the acid-base dissociation of dibucaine in aqueous media [5]. A pK value of 8.95 for the tertiary amine was determined. Quantum yields of 0.25 for dibucaine in phosphate buffer pH 7.0 and of 0.033 in KOH pH 12.6 were measured.

In our work, the pH dependence of dibucaine fluorescence was used to study the acid-base dissociation of dibucaine in micelles. Initially, control experiments in the absence of detergents were performed. The investigation was focused on the dissociation of the tertiary amino group of dibucaine corresponding to the transition from the monocation to neutral species.

The fluorescence emission spectra of dibucaine in aqueous media at different pH are shown in Fig. 2. A slight blue shift occurs upon going from acidic to alkaline media, as shown by the displacement of the emission peak. The fluorescence intensity exhibits, however, much stronger changes with pH. pH titrations were performed for $2 \cdot 10^{-5}$ M dibucaine in BCP buffer, and the integrated fluorescence intensity, A, was obtained for each pH value.

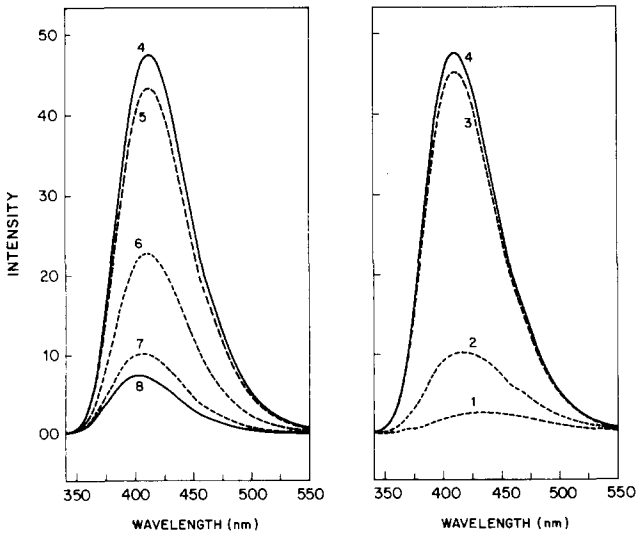

Fig. 2. Fluorescence emission spectra of dibucaine in 0.067 M borate-citrate-phosphate buffer. The pH values are: (1) 0.91, (2) 1.55, (3) 4.05, (4) 5.70, (5) 8.10, (6) 9.22, (7) 10.18, (8) 11.75. Excitation wavelength: 328 nm.

The quantum yield of each ionization state of dibucaine is related to the integrated fluorescence intensity. Using fluorescein as a standard, as described in Materials and methods, the values of 0.27 and 0.040 were obtained for the quantum yields of the monoprotonated and neutral dibucaine in buffer, respectively. The results of the pH titrations are presented in Fig. 3 (closed circles), where the values of the measured integrated intensities were transformed into quantum yields. Eq. (7) gives the best fit to the data for a pK of 9.0.

Solubilization of dibucaine in micellar systems such as the cationic CTAC, the anionic SDS, and the zwitterionic LPC detergents induces changes in its fluorescence emission spectrum. Titrations of $2 \cdot 10^{-5}$ M dibucaine with these detergents (results described in the following section) were used to obtain convenient values for the detergent concentrations, which allows one to consider that all dibucaine molecules are bound to the micelles. The pH titrations were then performed with dibucaine solubilized in BCP buffer containing 150 mM CTAC, 17 mM LPC, or 15 mM SDS. These are saturating detergent concentrations for 0.02 mM dibucaine since no further changes are observed upon adding more detergent.

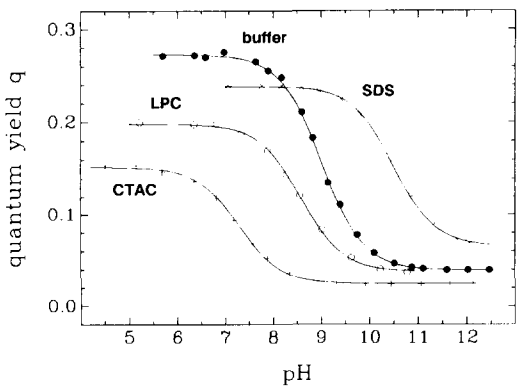

Fig. 3. Apparent quantum yield q (see Eq. (5)) of dibucaine in (●) buffer, (+) CTAC, (○) LPC, and (×) SDS, as a function of pH. The lines are best fits for the data using Eq. (7), for the acid-base dissociation equilibrium.

Table 1
Quantum yields of dibucaine in the protonated and neutral forms in different micellar environments and apparent pK values

	Quantum yield		pK_a	ΔpK_a	$e \cdot \psi$ (10^{-3} eV)
	D^+	D^0			
Buffer	0.27	0.040	9.00		
CTAC	0.15	0.024	7.29	−1.7	100
LPC	0.20	0.037	8.60	−0.4	23
SDS	0.24	0.066	10.52	1.5	−88

pK_a shifts for dibucaine in CTAC, LPC, and SDS micelles, ΔpK_a, relative to dibucaine in buffer, and their energy equivalent, $e \cdot \psi$, obtained from the expression $(-2.3 \cdot kT \cdot \Delta pK)$.

The emission spectrum of dibucaine in each of the three micellar systems were obtained as a function of the pH. The integrated intensities were normalized to the quantum yield of the monoprotonated dibucaine in buffer. The results are presented as plots of quantum yield q vs. pH in Fig. 3. Fitting the data using Eq. (7) gave the values of pK_a, the apparent pK for dibucaine in the micellar systems, and the quantum yields of both the protonated and the neutral forms of dibucaine in these systems. The obtained values are listed in Table 1.

The pK_a of dibucaine in LPC micelles is 8.6. This downward shift of 0.4 units with respect to the value in solution can be due to the lower dielectric constant of the micelle surface but an influence of a possible electric potential due to the positive and negative electric charges of LPC cannot be discarded. In the presence of the cationic micelles (CTAC) the aqueous pK_a is shifted downward to 7.3, whilst in the presence of the anionic micelles (SDS) it is shifted upward to 10.5.

The apparent pK shifts, ΔpK_a, induced by the micelles on dibucaine are both due to an intrinsic shift, ΔpK_i, upon dibucaine binding, and to the difference in pH values between the micelle surface and the bulk solution caused by micelle charges [1,20,21]. The Boltzmann equation gives the relationship which exists between the bulk and the surface concentration of protons for a given potential ψ_o:

$$[H^+]_o = [H^+]_b \exp[-e \cdot \psi_o / kT] \quad (11)$$

where e is the elementary electronic charge, k is the Boltzmann constant, and T is the absolute temperature. This equation leads to the following relationship,

which exists between the intrinsic and apparent pK shifts:

$$\Delta pK_a = \Delta pK_i - e \cdot \psi_o / 2.3kT \quad (12)$$

The values of ΔpK_a and of the energy contribution $e \cdot \psi = -2.3kT \cdot \Delta pK$, which includes both effects of the intrinsic shift and the potential ψ_o, are also in Table 1.

From these shifts it is possible to estimate the electric potentials affecting the tertiary amine of dibucaine. The values are 77 mV for CTAC and −111 mV for SDS, and were calculated from the values of $e \cdot \psi$ in Table 1, assuming that the intrinsic pK shifts are the same for the three micellar systems. It is interesting to compare these potentials with those obtained for tetracaine [4]. Tetracaine's aliphatic amino group is affected by potentials of 41 mV and −137 mV in the presence of cationic and anionic micelles, respectively. However the pK_a shifts for tetracaine used to calculate these potentials were relative to the pK measured in the neutral Triton X-100 micelles, while pK shifts relative to zwitterionic LPC were used in this work. Since surface potentials of 155 mV and −125 mV had been reported for CTAB and SDS micelles [4], it was concluded that tetracaine in cationic micelles is displaced towards the aqueous phase. For dibucaine, a similar conclusion is reached.

Binding of dibucaine to micellar systems

In addition to the sensitivity of the emission spectrum of dibucaine on the dissociation state of the molecule there is also a sensitivity to the micellar environment in which it is located. To monitor the binding of each dibucaine species to micelles of CTAC, LPC, and SDS we used the titration method in which the detergent concentration is varied at constant probe concentration. To meet a decision about which pH is convenient to guarantee the presence of only one ionization state of the drug we used the results of the pH titrations. The curves in Fig. 3 show directly that only protonated species occur at pH 5.5. They also show that to have only the neutral form of dibucaine a pH above 10.5 is convenient for CTAC and LPC, but for SDS the convenient pH is above 12.

Fig. 4 shows the changes of the peak wavelength and of the integrated intensity of the emission band for both the protonated (pH 5.5) and the neutral (pH 10.5 for CTAC, 11.5 for LPC and 12.2 for SDS) forms of dibucaine occurring upon addition of the detergents. A blue shift occurs upon incorporation of both forms of dibucaine into the micelles. In the three micellar environments the peak wavelength of the neutral form of dibucaine is lower than that of the protonated form. For the same ionization state the blue shift decreases in the order CTAC > LPC > SDS. For the neutral species in CTAC and LPC micelles both λ_{max} and A attain the saturation level at lower detergent concentrations than for the protonated one. This means that the binding constants of the neutral species are higher than the protonated ones. Below the critical micelle concentrations of these two detergents no spectral changes were observed. This is different with SDS micelles. Figs. 4C and F show that both the protonated and neutral forms of dibucaine interact with SDS at concentrations well below its cmc of 8 mM. The cmc of SDS lowers to a value around 2 mM. Moreover, with the protonated form the saturation level is reached at lower SDS concentrations than with the neutral form. This is a consequence of the opposite electric charges of SDS and protonated dibucaine. Interaction of dibucaine with lithium dodecyl sulphate below the cmc was already observed in low temperature fluorescence studies [6].

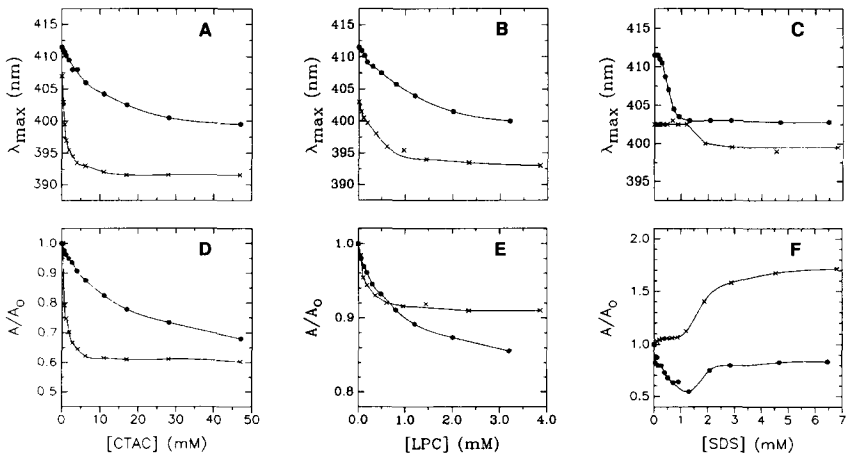

Fig. 4. Peak wavelength, λ_{max}, and integrated intensity normalized to the initial value, A/A_o, of the emission band of the (●) protonated (pH 5.5), and the (×) neutral (pH 10.5 for CTAC, 11.5 for LPC and 12.2 for SDS) forms of dibucaine as a function of detergent concentration: (A, D) CTAC; (B, E) LPC; (C, F) SDS.

The results of the titrations with detergents were used to find the binding constants of both forms of dibucaine to the micelles of CTAC, LPC and SDS. Referring to the emission spectra normalized to the same integrated intensity, the blue shift occurring with detergent addition makes the fluorescence intensity decrease at the right side of the spectrum (wavelengths above the peak) and increase at the left site (wavelengths below the peak). The wavelengths of 382 nm and 435 nm were chosen to obtain maximal fluorescence changes (see Materials and methods). The normalized fluorescence intensities at these two wavelengths were taken as spectral parameters for calculating the binding constants. The results are presented in Fig. 5 as plots of $(\varepsilon' - 1)$ vs. $(\varepsilon' - 1)/[M]$, as described in Materials and methods.

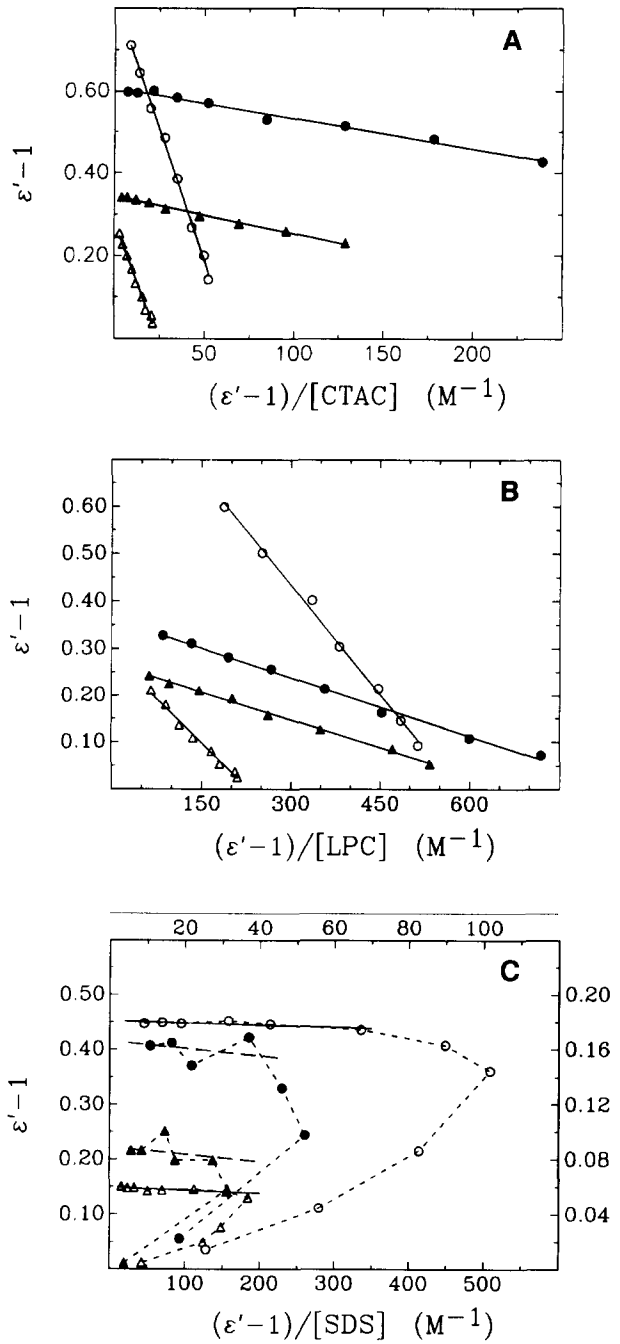

Fig. 5. Plots $(\varepsilon' - 1)$ vs. $(\varepsilon' - 1)/[M]$, where [M] is the concentration of (A) CTAC, (B) LPC, or (C) SDS. The values of the spectral parameter ε' defined in Materials and methods were obtained from the normalized fluorescence intensities at 382 nm (circles) and 435 nm (triangles). The lines are least-square fits for the data. The open simbols correspond to protonated dibucaine (pH 5.5) and the full symbols to neutral dibucaine (pH 10.5 for CTAC, 11.5 for LPC and 12.2 for SDS). The top and right scales in C correspond to the data of neutral dibucaine presented as full symbols.

For CTAC and LPC the plots are linear. The slopes of the best-fit lines in Fig. 5 give the values of the binding constants listed in Table 2. For SDS the curves have a more complex shape probably as a consequence of the interaction of dibucaine with monomers and pre-micellar aggregates, absent in CTAC and LPC. The binding constants to SDS micelles were obtained using the data for the highest concentrations. In the case of monoprotonated dibucaine the last 6 titration points were used to fit the straight lines. In the case of neutral dibucaine the scattering of experimental points does not allow a reasonable estimate of K_b (Fig. 5C). So the broken lines were drawn in the figure assuming that the value of K_b is essentially the same as that for CTAC and LPC.

The apparent binding constants presented in Table 2 were calculated from the slopes of the straight lines in Fig. 5, according to Eq. (10). The parallelism between the lines corresponding to the same system at two different wavelengths reflects the independence of the method on the wavelengths chosen. The values of K_b in Table 2 are obtained with the average of the two slopes. For D^+ in SDS the slopes only give a lower limit for K_b.

In the case of the neutral drug, D^0, the values of K_b for CTAC and LPC were very similar ($2.0 \cdot 10^3$ and $2.6 \cdot 10^3$ M^{-1}). For SDS, the broken lines obtained using $K_b = 2.3 \cdot 10^3$ M^{-1} give reasonable fits for the experimental data. Then, we conclude that the binding of D^0 to all the three micelles is very similar. On the other hand, for the protonated drug, D^+, the effect of micelle charge is very important: K_b for binding to CTAC is more than 6-fold smaller than that for LPC, while for SDS it is at least one order of magnitude greater.

The ratio of the apparent binding constants of the protonated and neutral dibucaine, K_b^+ to K_b^0, are related to the changes in the pK values of the drug [20,21]. For the intrinsic constants

$$K_{bi}^+ / K_{bi}^0 = \exp(2.3 \Delta pK_i) \quad (13)$$

where K_{bi}^+ and K_{bi}^0 are the intrinsic binding constants of the two forms of the drug, and ΔpK_i is the shift in

Table 2
Apparent binding constants of the protonated, K_b^+, and the neutral, K_b^0, forms of dibucaine to micelles of CTAC, LPC and SDS, obtained from the experimental data in Fig. 5

	K_b^+ (M^{-1})	K_b^0 (M^{-1})	ΔpK_{calc}	K_{bcalc}^+ (M^{-1})
CTAC	$1.5 \cdot 10^2$	$2.0 \cdot 10^3$	−1.1	40
LPC	$9.7 \cdot 10^2$	$2.6 \cdot 10^3$	−0.43	$1.0 \cdot 10^3$
SDS	$> 2.5 \cdot 10^4$	$(2.3 \cdot 10^3)$	(>1.0)	$7.3 \cdot 10^4$

ΔpK_{calc} is calculated by the expression $[\log(K_b^+ / K_b^0)]$. K_{bcalc}^+ is the binding constants of protonated dibucaine calculated from K_b^0 and the pK shifts of Table 1.

the intrinsic pK. If the concentration of D^+ at the membrane surface is given by the Boltzmann equation

$$[D^+]_{x=0} = [D^+]_b \exp(-e \cdot \psi_o / kT) \quad (14)$$

the ratio of the apparent binding constants is

$$K_b^+ / K_b^0 = \exp(2.3 \Delta pK_i - e \cdot \psi_o / kT) \quad (15)$$

Eqs. (12) and (15) show that the apparent change in pK upon micelle binding (values listed in Table 1) should be equal to $\log [K_b^+ / K_b^0]$ (ΔpK_{calc} values in Table 2). For LPC the values are indeed very close. For SDS there is just a compatibility between the two results. For CTAC, however, the experimental errors cannot account for the difference between the two values. It should be stressed that when using Boltzmann equations (11) and (14), it is assumed that both chemical potentials are the same in the bulk and in the interfacial phase [20]. Our results suggest that this is not the case for dibucaine. In other words, the difference in energy, which maintains a difference between the surface and bulk concentrations of H^+ (Eq. (11)), is not the same as that which maintains the difference between the concentrations of D^+ (Eq. (14)). The positive surface charge distribution of CTAC micelles is more effective in maintaining H^+ ions than D^+ away from the surface.

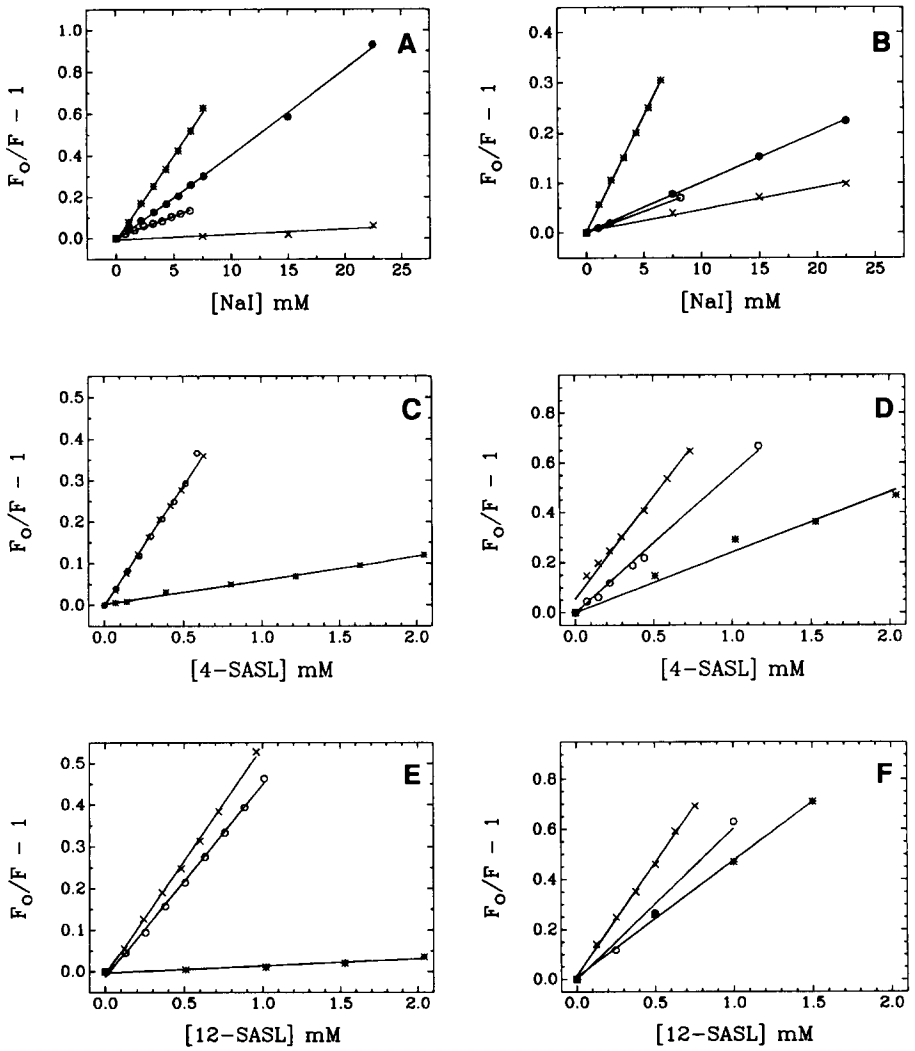

Fig. 6. Stern-Volmer plots for the quenching of dibucaine fluorescence by iodide (A, B), 4-SASL (C, D), and 12-SASL (E, F). •, Buffer; *, CTAC; ○, LPC; and ×, SDS. Plots on the left (A,C, and E) refer to protonated dibucaine (pH 5.5), and those on the right (B, D, and F), to neutral dibucaine (pH 10.5) for CTAC, 11.5 for LPC and 12.2 for SDS. The lines are least-square fits for the data.

Table 2 also lists the binding constants of protonated dibucaine, $K^+_{b\,calc}$, calculated from K^0_b and the experimental pK shifts of Table 1. Comparing these values with the experimental ones, for CTAC there is an almost 4-fold overestimation. For SDS, however, experiments only gave a lower limit for K^+_b. $K^+_{b\,calc}$ is, therefore, likely to give a better estimate. On the basis of CTAC results we would say that K^+_b for SDS is even greater than $K^+_{b\,calc}$.

Quenching of dibucaine fluorescence by Iodide, 4-SASL and 12-SASL

Iodide is an anion and do not penetrate the hydrophobic core of micelles. On the contrary, the stearic acids have a very high micelle/water partition coefficient and the number of molecules in the aqueous solution is negligible [22]. For these reasons these quenchers were used to characterize the sites of dibucaine in micelles.

Stern-Volmer plots of the quenching of protonated and neutral forms of dibucaine bound to each of the three micellar systems under consideration are presented in Fig. 6. The plots are linear in the concentration range covered by the experiments. This is not, however, an indication of occurrence of a single quenching process. It should be stressed here that the experimental errors for quenching of D^0 in CTAC and LPC by the SASLs are very high due to the low quantum yields and the presence of fluorescent impurities in the quencher solutions.

The slopes of the Stern-Volmer plots give the constants K_{SV}. The K_{SV} values obtained from the data in Fig. 6 are presented in Table 3. The fluorescence lifetimes for D^+ and D^0 in aqueous media obtained by [8], as well as the values in the micelles, calculated from the aqueous values and the quantum yields in the different micelles [23] also appear in Table 3.

For dibucaine in buffer, with respect to the quenching by iodide, the Stern-Volmer constant is four times greater for D^+ than for D^0. This seems to be a consequence of the negative charge of iodide. On the other hand, the bimolecular quenching contants calculated assuming pure colisional quenching ($1.2 \cdot 10^{10}$ M^{-1} s^{-1} for D^+ and $1.6 \cdot 10^{10}$ M^{-1} s^{-1} for D^0) are similar. A bimolecular quenching constant of $1 \cdot 10^{10}$ $M^{-1}s^{-1}$ may be considered as the largest possible value in aqueous solution [23]. This is an indication that there must be a contribution due to static quenching of dibucaine fluorescence by iodide. With respect to quenching by the SASLs, the low solubility in the aqueous phase makes it impossible to obtain the Stern-Volmer constants in buffer. The quenching processes in membranes are static in many cases. Even for dynamic quenching the bimolecular quenching constants would be very different from the values in buffer because diffusion in micelles is slower than in water. For these reasons only qualitative conclusions will be drawn from our quenching results.

In the case of dibucaine bound to CTAC, the K_{SV} values for iodide show that both D^+ and D^0 are more accessible to iodide than in buffer. This result aggrees with the cationic nature of the CTAC micelles. Since iodide is probably acting as counterions and penetrating into the region occupied by the micelle charges, we conclude that both dibucaine species are accessible to ions in the diffuse ionic double-layer of the micelle-buffer interface. With respect to quenching by the SASLs, D^+ in CTAC presents very low K_{SV} as compared to the other micellar systems, specially for 12-SASL. This confirms its ionic double-layer site. On the other hand, quenching of D^0 by the SASLs is quite effective and indicates a location in the hydrophobic core. We conclude that D^0 is solubilized both in the hydrophobic core and in the micelle surface.

In the case of LPC, there is a reduction of the accessibility of D^+ to iodide relative to buffer, but the accessibility of D^0 is about the same. The reduction in the accessibility of D^+ can be due to a screening of the positive charge of dibucaine by a redistribution of the dipolar charges of the LPC headgroups. The quenching by 4-SASL is more effective than by 12-SASL both for D^+ and D^0. We propose a similar location for both dibucaine species in LPC, with the aromatic rings in the hydrophobic portion and the tertiary amine in the micelle surface.

In the case of SDS the anionic nature of the micelle leads to a reduction of K_{SV} due to charge repulsion of iodide by the micelle surface. For neutral dibucaine,

Table 3
Stern-Volmer constants K_{SV} (M^{-1}) for the quenching of charged and neutral dibucaine fluorescence by sodium iodide, and by 4- and 12-SASL, in micelles of CTAC, LPC and SDS

	Iodide		4-SASL		12-SASL		τ_f (ns)	
	D^+	D^0	D^+	D^0	D^+	D^0	D^+	D^0
Buffer	40	10					3.3	0.6
CTAC	80	47	59 (1.9)	240 (7.7)	15 (0.5)	220 (7.3)	1.8	0.4
LPC	22	8.7	570 (3.2)	550 (3.1)	440 (2.5)	320 (1.8)	2.4	0.6
SDS	2.2	4.6	570 (2.4)	930 (3.9)	540 (2.3)	930 (3.9)	2.9	1.0

Numbers in parentheses were calculated using the local intramicellar concentration of the quencher. The fluorescence lifetimes τ_f in aqueous solution, obtained by [5] and in micelles, calculated using the quantum yields of Table 1, are also listed.

D^0, K_{SV} is reduced approximately twice relative to the same species in buffer, while for the monoprotonated drug, D^+, the reduction is much greater (18-fold). This would imply that D^+ is deeper in the interface than D^0. Quenching by 4- and 12-SASL are about the same both for D^+ and D^0. This would imply that dibucaine average distance from the 4th and 12th carbon atom of the hydrophobic chains are the same or that the nitroxide radicals of 4-and 12-SASL are constrained to the same average depth in SDS micelles.

Previous work on dibucaine in micelles [6] did not properly consider the amount of different species, D^+ and D^0, in aqueous and micellar phases, and for this reason the work has lots of unreliable considerations and conclusions. For example, the conclusion that dibucaine only exists as a free-base in neutral micelles at pH 5.5, 7.0 and 8.0 is wrong. According to the curve for LPC in Fig. 3, it exists as D^+ up to pH 7.5. Their conclusion that in cationic micelles D^+ appears to be situated in the Stern layer while D^0 is solubilized at the micellar interface came from experiments at 9.5 mM hexadecyltrimethylammonium bromide, where at least 40% of dibucaine molecules are not bound. Furthermore, there is no difference between their results at pH 7.0 and 8.0. Their conclusion seems, therefore, to be for aqueous and bound dibucaine, and not for bound D^+ and D^0. Their results on lithium dodecyl sulfate micelles were obtained under conditions such that only D^+ species were present. So, a conclusion about the location of D^0 in anionic micelles could not have been reached. These considerations show that quantitative results on the ionization equilibrium of bound drugs and on their binding constants are extremely important in obtaining reliable conclusions.

4. Conclusions

Dibucaine fluorescence provide a high sensitivity tool for investigating its interaction with membrane systems in the micromolar range.

The pK_a of dibucaine in water is 9.0. It is shifted to 8.6 in the presence of zwitterionic micelles of LPC. The apparent binding constants of charged and neutral dibucaine to LPC micelles were found to be $9.7 \cdot 10^2$ M^{-1} and $2.6 \cdot 10^3$ M^{-1}, respectively. The shift in pK_a is in complete agreement with the ratio of these binding constants. A practical consequence is that for neutral membranes the use of pK_a shifts to find the binding constant of one species from the value of the other is reliable. It should be noticed that for LPC micelles the binding constants only differ by a factor of the order of 3. This is apparently unexpected because values of water-egg PC partition coefficients for charged and neutral forms of local anesthetics differing by more than one order of magnitude have been reported [2,3]. However, the partition coefficients of the charged form in [2] were probably obtained under high drug concentration and include, therefore, the effect of the built up potential due to the binding of the positive drug to the neutral surface.

In the presence of the positively charged micelles of CTAC the pK_a is 7.3, which represents a downward shift of 1.7 relative to the aqueous pK. The values of the apparent binding constants of the charged and neutral dibucaine are $1.5 \cdot 10^2$ M^{-1} and $2.0 \cdot 10^3$ M^{-1}, respectively. The ratio of these constants can only account for a pK_a downward shift of 1.1. We conclude that the theoretical approach, which considers the charged species a punctual charge, and uses Boltzmann equation to correct its concentration in the solution immediately adjacent to the charged membrane surface, is poor. The use of pK_a shift to find the ratio of binding constants of neutral to charged species can lead to a 4-fold overestimation.

Anionic SDS micelles shift dibucaine pK_a to 10.5. The experimental data for neutral dibucaine agrees with a binding constant around $2.3 \cdot 10^3$ M^{-1}. Only an inferior limit of $2.5 \cdot 10^4$ M^{-1} was obtained for the protonated form. The restrictions imposed by the experimental data allowed, therefore, the limited conclusion that there is no contradiction between the pK_a shift and the values for the apparent binding contants obtained by titrations with SDS.

Apparent binding constants for the neutral form of the drug is approximately independent of the micelle charge, and they are not very sensitive to the hydrophobic tails of the detergents forming the micelles. This result allows using the same value for the binding constant of a neutral anesthetic species to all micellar systems. Furthermore, the observed independence is expected to be also valid for charged and neutral lipid vesicles. For the charged anesthetic the dependence is strong. Estimating the binding contants for the cationic form from that of the neutral form and from the pK shift gives excelent agreement for neutral micelles. For charged micelles, however, we have to take into account that the value is underestimated for positively charged micelles and also, probably, for negatively charged ones.

The quenching experiments allowed to reach some conclusions about the localization of dibucaine in micelles. The protonated dibucaine is completely immersed in the ionic diffuse double-layer of the CTAC micelle-buffer interface; in LPC the aromatic rings penetrate the hydrophobic region, and in SDS the molecule is even deeper than in LPC. These locations agree with the results of pK shifts which show that in CTAC micelles the magnitude of the potential affecting the tertiary amine of dibucaine is lower than that in SDS micelles. Mertz and Lin [6] achieved similar conclusions but analysis of their experimental conditions

on the basis of our results for pK shifts and binding constants shows that a wrong distribution of bound and free, charged and neutral dibucaine was assumed. The similarity is, therefore, accidental. Neutral dibucaine seems to have no preference for the core or the micelle surface. Fluorescence lifetime measurements would give more information about the quenching processes.

5. Acknowledgements

One of the authors (S.R.W.L.) would like to thank the kind and friendly reception during her stay at IFQSC. The authors acknowledge the financial support of FAPESP, FINEP, CNPq and MCT.

6. References

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