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THE EFFECT OF NEUTRAL AND CHARGED MICELLES ON THE ACID-BASE DISSOCIATION OF THE LOCAL ANESTHETIC TETRACAINE

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The influence of surfactant micelles on the acid-base dissociation of the charged tertiary amino group of the local anesthetic, tetracaine, has been investigated. From measurements of tetracaine fluorescence as a function of bulk pH, apparent p*K* values of 6.88, 7.58 and 9.92 were found in the presence of cationic, neutral and anionic micelles, respectively, in 10 mM NaCl. These values are considerably displaced with respect to the p*K* in aqueous solution which is 8.26. Such large shifts can be attributed to the effect of the surface polarity and electrical potential on the dissociation behavior of the anesthetic bound to micelles. It can be expected that the acid-base dissociation of a local anesthetic adsorbed to nerve fibers will also be affected by the properties of the membrane surface. Thus, it is suggested that the influence of the interfacial region on the p*K* of surface-bound molecules should not be disregarded when estimating the proportion of charged and uncharged forms of local anesthetics interacting with axonal membranes.

Introduction

The chemical structure of local anesthetics includes, in most cases, a secondary or tertiary amino group, the acid-base dissociation of which takes place near the physiological pH range [1]. Because of the presence of this dissociable group, the question has been raised of which species, the cation or the uncharged molecule, is responsible for the pharmacological effect [1,2]. It is now widely accepted that whereas only the neutral molecule is freely permeable through the nerve, it is the cation the form which is active, the site of its action being the internal surface of the axonal membrane [1]. This hypothesis is supported by a number of investigations of the pH dependence of the blocking potency of several dissociable anesthetics applied to nerve fibers and to giant squid axons either internally or externally [3–6].

In the above-mentioned studies, the determination of the blocking potency of an anesthetic requires a correct estimation of the actual concentration of the active form of the drug, at a certain pH. For this purpose, the ratio between the charged and uncharged anesthetic forms is always calculated by means of the Henderson-Hasselbach equation in which the aqueous p*K* of the drug and the bulk pH of the anesthetic solution, are inserted [2–7]. Local anesthetics, however, bind to membranes [8–13] and the apparent dissociation constant of a molecule located at an interfacial region may be very different from the dissociation constant of the same molecule in bulk solution [14–16]. Thus, we decided to study the acid-base dissociation of a local anesthetic in the presence of micelles in order to determine to what extent the interfacial properties modify the apparent p*K* of the surface-

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Abbreviation: CTAB, cetyltrimethylammonium bromide.

bound drug. Tetracaine, a molecule that binds strongly to membranes [8–13], was chosen as representative of dissociable local anesthetics and detergent micelles of different charge were taken as simple models of the interfacial region of biological membranes. In order to perform this investigation we designed a fluorescence method which allows to determine the dissociation constant using tetracaine concentrations as low as 3 μM, such that the effect of the cationic anesthetic form on the charge density of the model membranes employed, can be neglected. In addition, this value is in the range of the concentrations required to block conduction in isolated nerve fibers [2,6]. A preliminary account of this work has been presented elsewhere [17].

Materials and Methods

Crystalline tetracaine-HCl (2-(dimethylamino)ethyl-4-(*n*-butylamino)benzoate hydrochloride) was obtained from Sigma. Triton X-100, sodium dodecyl sulfate and cetyltrimethylammonium bromide were purchased from BDH. Spectroscopic grade dioxane (UVASOL) was from Merck. All other reagents were analytical grade. Ultrapure water obtained through a Milli RO-Milli Q purification system of Millipore, was used throughout.

The detergent solutions were prepared in aqueous 10 mM NaCl. Unless otherwise noted, the surfactant concentrations were 10 mM, 17 mM and 10 mM for Triton X-100, sodium dodecyl sulfate and cetyltrimethylammonium bromide, respectively. Dioxane/water mixtures of different polarity were prepared at 20, 45, 70 and 82 wt% dioxane to give dielectric constants of 60.79, 38.48, 19.69 and 9.53, respectively [18].

Excitation and emission spectra as well as fluorescence measurements at constant wavelengths, were obtained in an Aminco-Bowman spectrophotofluorometer equipped with a Hewlett-Packard 7004 B Recorder. Slit widths equivalent to 5.5 nm bandpass were used throughout.

Titration of tetracaine fluorescence excited at 302 nm and emitted at 360 nm, was carried out by adding small aliquots of concentrated HCl or NaOH solutions to the aqueous, partially non-aqueous or micellar samples containing 3 μM anesthetic. Fluorescence was read immmediately after recording pH with a Radiometer pH meter Model 22 equipped with a glass-Ag/AgCl combination electrode.

The pH readings in the partially non-aqueous solutions were corrected as previously described by Van Uitert and Haas [19] and Fernández and Fromherz [16].

The experiments were repeated at least five times. Temperature was kept constant at 22 ± 1°C.

Results and Discussion

Determination of the acid-base dissociation constants of tetracaine amino groups by fluorescence titration

In Fig. 1 is illustrated the effect of pH on the uncorrected fluorescence spectra of tetracaine in 10 mM NaCl aqueous solution. The wavelength of maximum emission is not affected by pH whereas the excitation maximum is only slightly shifted towards the red as the pH is lowered. The flurorescence intensity, however, does exhibit a considerable change with pH: it is much higher at pH 12 than at pH 4.3 and becomes negligible at pH 1. In this regard it should be mentioned that it has

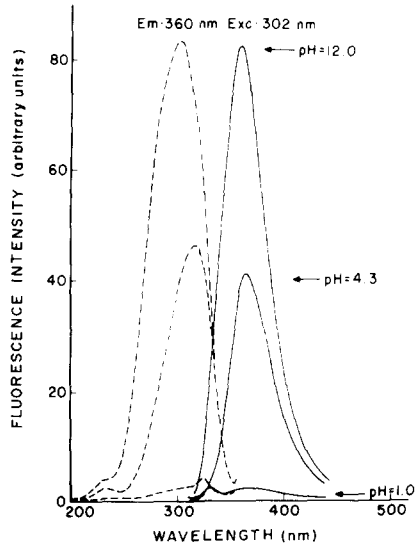

Fig. 1. The effect of pH on the fluorescence spectra of tetracaine. The anesthetic concentration was 3 μM in 10 mM NaCl aqueous solution. Excitation spectra (- - - - -); emission spectra (———).

already been reported that the fluorescence of tetracaine measured at constant wavelengths is 30% higher at pH 10 than at pH 7 [20].

To investigate what kind of function relates tetracaine fluorescence and pH, titration experiments were performed using constant wavelengths, as selected from the spectra in Fig. 1. Inspection of this figure shows that maximal changes in fluorescence with pH can be measured by setting the emission at 360 nm and the excitation at 302 nm. The curve resulting from the titration experiment (Fig. 2) exhibits two sigmoidal regions: one at pH values below 4 and the other between pH 7 and 10. Both regions have the shape predicted by the Henderson-Hasselbach equation for the pH dependence of the dissociation degree of weak acids [21]. Since tetracaine has two dissociable groups, a secondary aromatic amine and a tertiary aliphatic amine, the variation of fluorescence intensity with pH in each sigmoidal region can be attributed to titration of one of these two amines. According to this interpretation, the curve of Fig. 2 was normalized to represent the dissociation degree (α) of each charged amino group as a function of pH as shown in Fig. 3. By taking the pH corresponding to $\alpha = 0.5$ as equal to the pK, the curves yield pK values of 2.14 ± 0.02 (pK_1) and 8.26 ± 0.04 (pK_2). Since a charged aromatic amine is more acidic than a charged aliphatic amine [22], pK_1 should correspond to the first group and pK_2 to the second one (Fig. 4). That these pK values are correctly assigned and that they do indeed correspond to dissociation of the charged amino groups in the ground state, can be confirmed by comparison with results obtained by other methods.

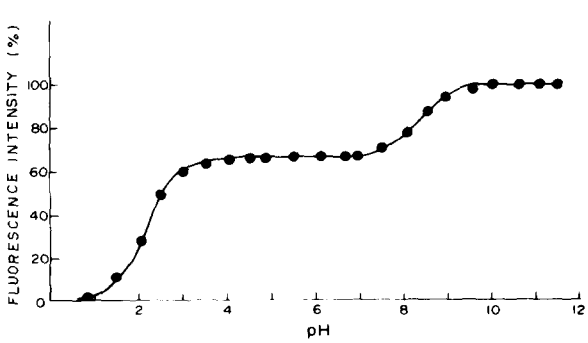

Fig. 2. Fluorescence intensity of tetracaine as a function of pH in aqueous solution. The medium contained 3 μM tetracaine and 10 mM NaCl. Excitation: 302 nm; emission: 360 nm.

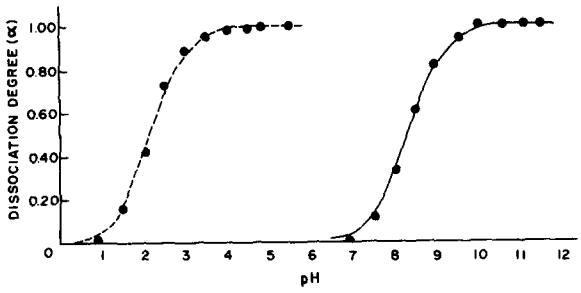

Fig. 3. Dissociation degree of the charged aromatic and aliphatic amino groups of tetracaine as a function of pH in aqueous solution. The medium contained 3 μM tetracaine and 10 mM NaCl. Aromatic amine (- - - - -); aliphatic amine (———).

For the aromatic amino group, potentiometric titration yields a pK of 2.25 (Fernández, M.S., unpublished). In addition, by use of ^{13}C-NMR Boulanger et al. [23] measured a pK of 1.95. Both values are close to the pK_1 reported here.

As for the aliphatic amine, by means of potentiometric titration Skou [24] found a pK of 8.24, Eisenbrand and Picher [25] reported a value of 8.47 whereas Boulanger et al. [23] detected a pK of 7.50, very low as compared to the results of the other authors. Our value of 8.26 is the same as that of Skou and very close to the result of Eisenbrand and Picher.

Since the values obtained by fluorescence titration agree with previous results, it can be concluded that the method reported herein allows to determine accurately the pK values of the aromatic and aliphatic amino groups of tetracaine in the ground state. Because of the high sensitivity of this method, titrations can be performed at anesthetic concentrations in the micromolar range in contrast to potentiometric [23–25] and NMR [23] methods which require millimolar concentrations to give reliable results.

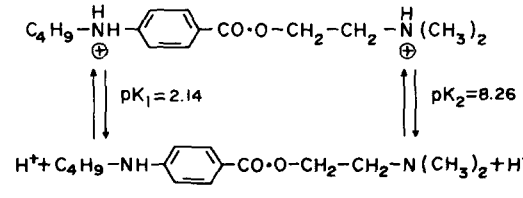

Fig. 4. Acid-base dissociation of the charged aromatic and aliphatic amino groups of tetracaine. The pK values shown were determined in aqueous 10 mM NaCl.

Of the two amino groups of tetracaine, it is the aliphatic amine the one which dissociates near the physiological pH range showing a pK of 8.26 (pK_2). Thus, the investigation of the effect of micelles on the dissociation behavior of tetracaine, was focused on this group.

The effect of micelles on the pK_2 tetracaine

Before carrying out the pH titration of tetracaine in the presence of micelles, the influence of the detergent concentration on the anesthetic fluorescence at constant pH was determined. Fig. 5 shows the relative fluorescence intensity of 3 μM tetracaine at pH 11 as a function of concentration for the neutral surfactant Triton X-100. It can be seen that up to 0.2 mM surfactant, the relative fluorescence intensity remains constant at the same value obtained in the absence of detergent. Above 0.2 mM, however, the fluorescence intensity increases with Triton X-100 concentration and becomes constant at concentrations higher than 5 mM. Taking into account that a critical micellar concentration of 0.238 mM has been reported for Triton X-100 [26], the results in Fig. 5 can be interpreted to mean that whereas the anesthetic fluorescence is not influenced at all by the monomeric detergent, it is strongly increased by micelles. Since it has been widely demonstrated that tetracaine binds to membranes [8–13], the fluorescence

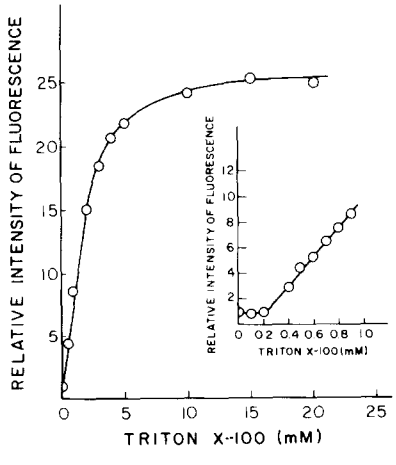

Fig. 5. Relative fluorescence intensity of tetracaine as a function of Triton X-100 concentration. The detergent was dissolved in aqueous 10 mM NaCl containing 3 μM anesthetic. The pH of the medium was kept constant at 11.00. Excitation: 302 nm; emission: 360 nm.

increase observed here may be attributed to partition of tetracaine at the micellar surface which, because of having a dielectric constant much lower than water [16], will enhance the quantum yield of the fluorophore [27]. Tetracaine fluorescence levels off above 5 mM Triton X-100 indicating that a saturating concentration of micelles with respect to the anesthetic concentration employed (3 μM) has been attained.

The dependence of tetracaine fluorescence with detergent concentration at constant pH was also studied using anionic sodium dodecyl sulfate (SDS) and cationic cetyltrimethylammonium bromide (CTAB) (not shown). In both cases it was found that fluorescence is also not modified by these detergents below their respective critical micellar concentrations but is enhanced in the presence of micelles, the fluorescence leveling off at detergent concentrations above 7 mM and 4 mM for SDS and CTAB, respectively.

In view of these results, the pH titrations of tetracaine in the presence of micelles were carried out using solutions of Triton X-100, SDS and CTAB at 10 mM, 17 mM and 10 mM, respectively. All these values are in the leveling off region of the corresponding fluorescence versus concentration curves. In addition, the concentration chosen for each detergent is about 9–10 mM above its critical micellar value [26–28].

Considering the aggregation numbers of the micelles [29] and the concentration of tetracaine employed (3 μM) it can be calculated that the ratio between the number of anesthetic molecules and the number of micelles is 1 : 48, 1 : 50 and 1 : 300 for SDS, CTAB and Triton X-100, respectively. This implies that there is much less than one molecule of tetracaine available per micelle. It can be concluded that in the titration experiments of tetracaine in the presence of micelles, the influence of the charged form of the anesthetic on the micellar surface potential, can be disregarded.

The same procedure described for the aqueous solution was employed to determine the influence of micelles on the pK_2 of tetracaine in 10 mM NaCl solutions. The curves of dissociation degree (α) versus pH resulting from these experiments are shown in Fig. 6. From these plots, apparent pK_2 values of 6.88 $\pm$ 0.07, 7.58 $\pm$ 0.05 and 9.92 $\pm$ 0.05 are found in the presence of cationic, neutral and

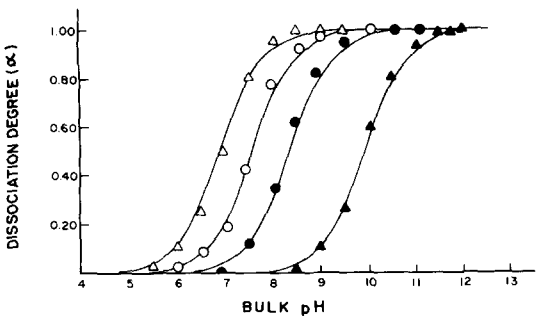

Fig. 6. Dissociation degree as a function of pH for the charged aliphatic amino group of tetracaine in the presence of micelles. Neutral micelles of Triton X-100 (○); cationic micelles of CTAB (△); anionic micelles of SDS (▲); no detergent present (●). The curves shown were obtained by normalizing the corresponding plots of tetracaine fluorescence (excitation 302 nm, emission 360 nm) versus pH. Detergent concentrations were 10 mM Triton X-100, 10 mM CTAB or 17 mM SDS. All samples contained 3 μM tetracaine and 10 mM NaCl.

TABLE I

THE EFFECT OF THE DIELECTRIC CONSTANT ON THE pK OF TETRACAINE ALIPHATIC AMINO GROUP (pK_2) IN DIOXANE-WATER SOLUTIONS

The pK_2 values shown, which are the means ±S.D. of five experiments, were calculated from the normalized curves of tetracaine fluorescence (excitation 302 nm, emission 360 nm) versus pH obtained for the dioxane/water solutions containing 3 μM tetracaine and 10 mM NaCl. The pH readings in these solutions were corrected as previously described [16,19]. The dielectric constants of the dioxane/water solutions were taken from Harned and Owen [18].

wt. % dioxane in dioxane/water mixtures	Dielectric constant	pK_2
0	81.07	8.26±0.04
20	60.79	7.85±0.07
45	38.48	7.25±0.07
70	19.69	7.19±0.08
82	9.53	7.09±0.04

anionic micelles, respectively. The large shifts induced by micelles on tetracaine pK_2 can be attributed to the effect of the interfacial properties on the dissociation of the anesthetic bound to micelles.

The pK_2 of tetracaine in water has a value of 8.26 which is shifted to 7.58 in the presence of neutral micelles of Triton X-100. This shift can be explained by the effect of the lower dielectric constant of the micellar surface [16,30] on the intrinsic pK. For the dissociation of a cationic acid such as the charged aliphatic amine of tetracaine, a lowering of the medium dielectric constant should lead to a decrease in the pK through stabilization of the uncharged base resulting from deprotonation of the acidic species [16,31]. This interpretation is strongly supported by the data of Table I, where the pK_2 values obtained by titration of tetracaine in dioxane/water solutions of different polarity, are shown. It can be observed that as the dielectric constant is lowered, the pK_2 decreases.

Concerning the effect of a neutral surface on the pK of adsorbed molecules, it should be mentioned that in a study of the influence of drugs on dipalmitoylphosphatidylcholine transition temperature [32], best fits between experimental data and a theoretical model were obtained by assuming that as a result of binding to neutral membranes the pK of procaine changes about one unit with respect to the value in solution whereas the pK change for tetracaine is close to zero. However, in view of the shift in tetracaine pK that we have obtained in the presence of Triton X-100 micelles, it could be expected that a significant change in the pK of this anesthetic will also be obtained due to adsorption to the neutral phosphatidylcholine liposomes.

As for the effect of charged surfaces on tetracaine dissociation we have found that in the presence of cationic or anionic micelles, the aqueous pK_2 of 8.26 is shifted to 6.88 and 9.92, respectively. These changes can be assigned to a shift of the intrinsic pK produced by the low interfacial polarity of micelles and to an additional shift induced by the surface electrical potential. The effect of the electrical potential can be described by the following equation [16,33]:

$$pK_{ch} = pK_0 - \frac{F\psi}{2.3\,RT} \qquad (1)$$

where pK_{ch} is the observed pK at the charged surface, pK_0 is the intrinsic pK at the same surface, ψ is the electrical potential, F and R are the Faraday and the gas constant, respectively, and T is the Kelvin temperature. Since it has been estimated that Triton X-100, SDS and CTAB micelles have similar surface dielectric constants ($\varepsilon \simeq 32$),

the intrinsic pK in the charged micelles can be taken as equal to the pK found in the neutral micelles [16]. Eqn. 1 shows that for a dissociation process taking place at a surface affected by a negative surface potential, the pK will be larger than at a neutral interface, while the opposite will hold true for a positively charged surface. Our results are in agreement with these predictions. Moreover, substituting in Eqn. 1 pK_{ch} by the pK_2 obtained in CTAB or SDS micelles, and pK_0 by the pK_2 measured in Triton X-100 micelles, it is possible to estimate the electrical potentials affecting the dissociation of tetracaine at the charged surfaces. In this way, it can be calculated that tetracaine aliphatic amino group is affected by surface potentials of +41 mV and −137 mV in the presence of cationic and anionic micelles, respectively. In addition, from the salt concentration dependence of the electrical potential of charged micelles detected with the dye 4-heptadecylumbelliferone [16], it is possible to calculate surface potentials of +155 mV and −125 mV for CTAB and SDS micelles, in 10 mM NaCl. There is a striking similarity between the potentials sensed by tetracaine and the dye in the anionic micelles while in cationic micelles, a smaller potential is detected by the anesthetic as compared to heptadecylumbelliferone. Taking into account that the magnitude of the potential decreases as the distance from the interface is increased in the direction of the aqueous phase, it can be suggested that the aliphatic amino group of tetracaine may be closer to the surface of SDS micelles as compared to its location in CTAB micelles. In the latter case the group could be displaced towards the aqueous phase, yet near enough the interfacial plane as to be affected by a positive surface potential of 40 mV.

We have demonstrated that both the surface dielectric constant and electrical potential have large influence on the acid-base dissociation of the aliphatic amino group of tetracaine interacting with micelles. Previous results from NMR studies on the interaction of tetracaine with liposomes [9,10,34] and micelles [12] suggest that while the aromatic moiety of the drug is located in the hydrophobic membrane region, the aliphatic dimethylamino group points towards the lipid/water interface. Our present findings are consistent with this proposal since such location explains the sensitivity of the dissociation behavior of the aliphatic amino group to the interfacial properties.

Taking into account that natural membranes bear negative surface charge [35] and, in particular, that there is a high density of negative surface charge near the sodium channel [36] which is the site of the anesthetic action, it could be presumed that the pK of a drug adsorbed onto that membrane region will not be the same as in solution. Thus, it is suggested that in the calculation of the proportion of charged and uncharged anesthetic forms interacting with axonal membranes, the errors involved in the usual assumption that equates the surface pK of the drug to its bulk pK, should not be overlooked.

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