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FORMATION OF MICELLES AND MEMBRANE ACTION OF THE LOCAL ANESTHETIC TETRACAINE HYDROCHLORIDE

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Summary

The formation of micelles of the local anesthetic tetracaine hydrochloride in aqueous phosphate buffer solution of pH 6.5 and ionic strength (I) 0.10 was examined at 22°C by surface tension and using the fluorescent indicators perylene (peri-dinaphthalene) and 8-anilino-1-naphthalene sulfonic acid, sodium salt (ANS). The critical micelle concentration was located at 0.069, 0.071 and 0.063 M by measurements of surface tension, perylene solubilization and enhancement of ANS fluorescence, respectively. In contrast to other cationic surfactants, the anesthetic monomer did not show evidence of forming a fluorescent molecular complex with ANS under the experimental conditions of this study.

The formation of micelles by tetracaine-HCl showed a pronounced effect on lipid membranes by inducing an abrupt decrease in the scattered light of egg lecithin liposomes at an anesthetic concentration roughly similar to its critical micelle concentration. This optical behaviour is characteristic of liposome damage and can be interpreted to mean that the lipids become solubilized into tetracaine-HCl micelles.

The ability of this local anesthetic to form micelles can be taken as a manifestation of the same hydrophobic forces that lead to partitioning of the drug into membranes.

Introduction

A primary factor for the local anesthetic activity of tetracaine-HCl is the ability of this drug to interact with membranes [1]. As a consequence, much work has been done to investigate the nature of this interaction [2,3]. Studies

using artificial model systems [4—6] have shown that hydrophobic binding plays an important role in the attachment of the drug to membranes. These findings can be correlated with the proposal that the membrane activity of a certain compound is closely dependent on its hydrophobic character [7].

The same hydrophobic forces that lead to partition of a lipophilic substance into a non-polar phase are also partially responsible for the cooperative self-association of many amphipathic compounds to form micelles [8]. Formation of micelles gives rise to abrupt changes in the concentration dependence of some physicochemical properties of amphiphilar solutions. A critical micelle concentration characterizing the transition from monomers to micelles can be determined on the basis of such changes [9,10].

The question as to whether amphipathic tetracaine-HCl has enough hydrophobic character to undergo micelle formation, has been raised several years ago. In this respect, the existence of a 'critical concentration' was first suggested by Rohman et al. [11] to explain the deviations shown by the freezing-point depression curve of tetracaine-HCl solutions, which appeared in the 1948 edition of the Danish Pharmacopoeia. Other investigators have found that, in fact, tetracaine-HCl forms aggregates as detected by measurements of equivalent conductance and osmotic coefficients [12]. However, they could not determine a critical micelle concentration because no defined break appeared when the properties under study were plotted versus the anesthetic concentration.

The purpose of the present work was to study the aggregation of tetracaine-HCl and to find out whether a critical micelle concentration could be detected by measurements of surface tension, solubilization of perylene (peridinaphthalene) and enhancement of 8-anilino-1-naphthalene sulfonic acid, sodium salt (ANS) fluorescence. In addition, the effect of the anesthetic self-association on the stability of artificial lipid membranes was investigated.

Materials and Methods

Crystalline tetracaine-HCl (2-dimethylaminoethyl-4-*n*-butylamino benzoate hydrochloride) and egg lecithin, were from Sigma Chemical Company. The anesthetic was purified by recrystallization from water and the lipid by column chromatography on alumina [13]. The purity of the lecithin obtained was checked by thin-layer chromatography. Perylene was purchased from Aldrich Chemical Co., 8-anilino-1-naphthalene sulfonic acid, sodium salt from Eastman Organic Chemicals and Brij 35 (poly(oxyethylene) lauryl ether) from BDH Chemicals Limited. All other reagents were analytical grade. Glass redistilled water was used throughout.

Unless otherwise stated, the aqueous solutions were prepared using a phosphate buffer (0.046 M KH_2PO_4/0.021 M Na_2HPO_4) of ionic strength (I) 0.10 and pH 6.5.

All the experiments were repeated at least seven times. The temperature was kept constant at 22 ± 1°C.

Surface tension measurements. Surface tension was determined by the ring detachment method [14] employing a Cenco-du Noüy tensiometer. The solutions, placed in dishes of 5.5 cm diameter, were allowed to equilibrate

for 10 min before doing the measurements.

Experiments using fluorescent indicators. To study micelle formation through solubilization of perylene by tetracaine-HCl solutions, very fine glass beads (200—250 Mesh) previously coated with perylene from an acetone solution [15] were used. In this way, the surface area of perylene in contact with the aqueous phase was enlarged. Solutions of various concentrations of the anesthetic in the buffer of pH 6.5 were made and 25 ml of each were placed in separate flasks containing 50 mg of glass beads that had been coated with 8 μg of perylene. The flasks were stoppered and shaken continuously for 20 h in the dark. After this time the beads were filtered off. The perylene solubilized in the filtrates was detected by measuring its fluorescence intensity after the addition of Brij 35 (final concentration 1%) and the lowering of the pH to 1.0 with concentrated HCl. This procedure was necessary for the fluorometric detection of perylene because, at pH 6.5, its fluorescence is quenched by the anesthetic whereas at pH 1.0, in the presence of a neutral detergent such as Brij 35, the quenching is almost completely abolished (Fernández, M.S., unpublished results). Under the experimental conditions employed, the highest concentration of perylene extracted into the aqueous phase was $6 \cdot 10^{-7}$ M.

For the studies of micelle formation through ANS fluorescence enhancement, the indicator was dissolved in aqueous buffer solutions of the anesthetic to give a final concentration of $2 \cdot 10^{-5}$ M.

Fluorescence measurements were made with an Aminco Bowman spectrophotofluorometer. Perylene fluorescence was excited at 409 nm and the emission was detected at 469 nm; for ANS the excitation was at 387 nm, the emission at 474 nm (uncorrected wavelengths). Excitation and emission slit widths equivalent to 5.5 nm bandpass were used.

Light-scattering determinations. Egg lecithin liposomes (mostly unilamellar) were prepared by sonication as previously described [5,16] followed by centrifugation at 100 000 $\times g$ for 1 h to eliminate the small proportion of large vesicles. To different aliquots of the dispersion, diluted to a final phosphatidylcholine concentration of $9 \cdot 10^{-4}$ M, the desired amounts of tetracaine-HCl were added. The 90° light-scattering of the lipid dispersions was measured at 600 nm with the Aminco Bowman spectrophotofluorometer.

Results

Determination of the critical micelle concentration

Figs. 1, 2 and 3 illustrate the concentration dependence of surface tension, fluorescence of solubilized perylene and ANS fluorescence for solutions of tetracaine-HCl in phosphate buffer of pH 6.5 and $I = 0.10$. The three plots show marked kinks in the region corresponding to 0.06—0.07 M tetracaine-HCl. In each case the critical micelle concentration is determined graphically as the anesthetic concentration corresponding to the point where the two extrapolated linear portions of the curve intersect [17,18].

(a) Surface tension measurements. In Fig. 1 is shown the plot of surface tension vs. logarithm of molar concentration for tetracaine-HCl aqueous solutions. Surface tension is lowered by increasing the anesthetic concentration to approx. 0.07 M. Further addition of anesthetic does not modify the surface

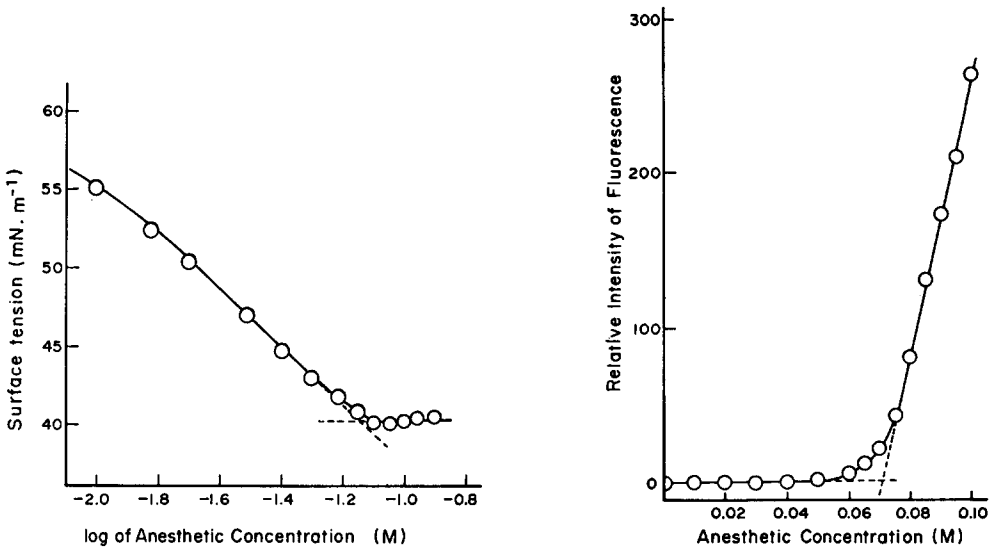

Fig. 1. Surface tension as a function of the logarithm of concentration for solutions of the anesthetic tetracaine-HCl in aqueous phosphate buffer of pH 6.5 and $I = 0.1$; $t = 22°C$.

Fig. 2. Fluorescence of perylene solubilized in the aqueous phase versus concentration of the anesthetic tetracaine-HCl. Perylene coated onto glass beads was extracted by anesthetic solutions prepared in phosphate buffer of pH 6.5 and $I = 0.1$ The fluorescence of the solutions was read at pH 1 in the presence of Brij 35; this procedure was to eliminate the quenching of perylene fluorescence induced by tetracaine at pH 6.5. Under the experimental conditions used, the highest concentration of perylene solubilized in the aqueous phase was $6 \cdot 10^{-7}$ M. Excitation and emission wavelengths were 409 nm and 469 nm, respectively (uncorrected values); $t = 22°C$.

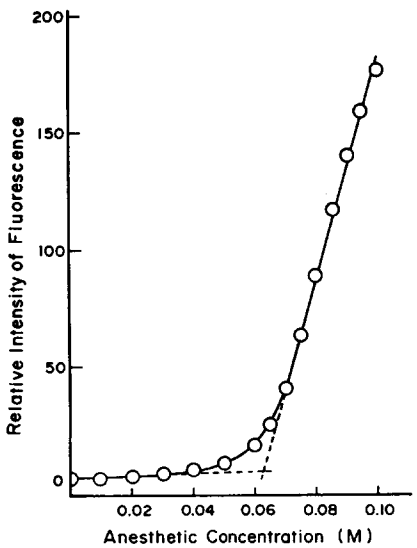

Fig. 3. Fluorescence of ANS as a function of concentration for solutions of the anesthetic tetracaine-HCl in aqueous phosphate buffer of pH 6.5 and $I = 0.1$. The ANS was used at a constant concentration of $2 \cdot 10^{-5}$ M. Fluorescence was excited at 387 nm and the emission detected at 474 nm (uncorrected wavelengths); $t = 22°C$.

tension. The portion of the curve showing a negative slope may be attributed, according to the Gibbs equation [19], to adsorption of the anesthetic monomer at the air/water interface. The plateau may be interpreted as due to formation of aggregates that precludes further interfacial adsorption of monomeric tetracaine-HCl. A critical micelle concentration of 0.069 M is found by this method.

(b) Solubilization of perylene. Tetracaine-HCl self-association has also been investigated in this work, by use of perylene as indicator of micelle formation. Mast and Haynes [15] have suggested recently that such a polycyclic aromatic compound could be employed to locate critical micelle concentrations. Perylene is extremely insoluble in water but is solubilized in micelles and due to its fluorescence is detected easily. In the present case of solubilization of perylene by tetracaine-HCl at pH 6.5, the fluorescence cannot be read directly because at this pH the anesthetic behaves as a quencher of perylene fluorescence. The quenching, however, is almost completely eliminated at pH 1.0 in the presence of a neutral detergent. Fig. 2 shows the fluorescence intensity of perylene solubilized by increasing concentrations of tetracaine-HCl. It can be seen that perylene becomes solubilized in the aqueous phase when the anesthetic concentration is higher than approx. 0.06 M. From the plot in Fig. 2 a critical micelle concentration of 0.071 M is obtained. It must be noted that, under the experimental conditions of this work, the maximum concentration of perylene dissolved by a tetracaine-HCl solution was $6 \cdot 10^{-7}$ M. This concentration is low enough for it to be considered that the solubilizate does indeed behave as an indicator and that it does not induce a perturbation in the aggregation process.

(c) Enhancement of ANS fluorescence. The third method employed to detect micelle formation consisted of the use of the polarity indicator, ANS. This probe is almost nonfluorescent in water, whereas it becomes strongly fluorescent when located in regions of lower polarity such as the micelle/water interface [15]. Fig. 3 shows the effect of increasing concentrations of tetracaine-HCl on ANS fluorescence. It can be seen that there is virtually no detectable fluorescence below 0.06 M tetracaine-HCl. Above this concentration, ANS fluorescence increases almost linearly as more anesthetic is added to the solution. By use of this method, the critical micelle concentration is located at 0.063 M, a value which does not differ significantly from the results obtained by surface tension or solubilization of perylene. This is important to be noted because under the experimental conditions of this work, the anesthetic monomer is present mainly in the cationic form and aminonaphthalene sulfonates such as ANS have been reported unsuitable for determining the critical micelle concentration of cationic surfactants [15]. It was reported, for example, that solutions of 8-toluidinyl naphthalene sulfonate and cetyl trimethylammonium bromide become opalescent and that the most abrupt change in fluorescence does not occur at the critical micelle concentration but at a detergent concentration equivalent to the concentration of the fluorescent probe. From these results it was concluded that a 1 : 1 fluorescent molecular complex is formed between the fluorescent probe and the detergent [20].

In the present work, ANS was used at a concentration of $2 \cdot 10^{-5}$ M. Thus, if the anesthetic monomer and the ANS were able to form a fluorescent molec-

ular complex in a way similar to that with cetyl trimethylammonium bromide and 8-toluidinyl naphthalene sulfonate, one would expect ANS fluorescence to be enhanced by the very first addition of tetracaine-HCl. Fig. 3 shows that this is not the case since the increase in ANS fluorescence appears in the region corresponding to 0.06 M tetracaine-HCl. In addition, it should be noted that samples of tetracaine-HCl plus ANS did not show turbidity under the experimental conditions of this work. In view of these observations, it can be concluded that the increase of ANS fluorescence shown in Fig. 3 is due to adsorption of the probe to the interfacial region of lower polarity which becomes available on aggregation of the anesthetic.

Effect of anesthetic aggregation on liposomes

To investigate whether tetracaine-HCl self-association has any effect on membranes, the influence of increasing concentrations of the anesthetic on the scattered light of lipid vesicles was examined. Fig. 4 illustrates how the 90° scattered light of $9 \cdot 10^{-4}$ M egg lecithin liposomes is modified as tetracaine-HCl is added to the dispersion. The scattering increases with the addition of anesthetic up to 0.050 M but it drops sharply at 0.060 M to remain at a value near zero above this concentration.

An increase in light-scattering is usually attributed to aggregation of liposomes [21]. On the other hand, a decrease can be accounted for by two different phenomena: lipid phase transition or liposome damage [21—24]. As for the first possibility, a tetracaine-induced phase transition can be excluded because light-scattering experiments were performed at 22° C, well above the temperature at which egg lecithin phase-change takes place (—15 to —5° C) [25]. Moreover, if any modification is induced by the presence of the anesthetic, it can be expected to be a lowering of the transition temperature [26]. Thus, the sharp reduction in scattering intensity shown in Fig. 4 should be taken as suggestive of liposome damage. In this respect it should be mentioned that vesicle damage can be followed by changes in light-scattering as efficiently as by measuring the leakage of markers entrapped in liposomes [22—24].

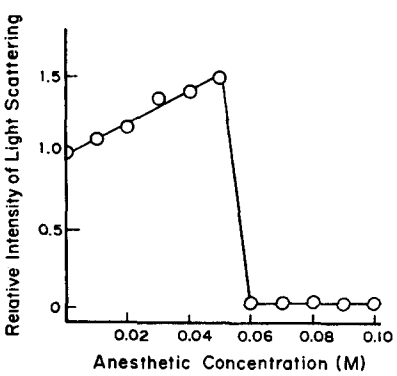

Fig. 4. The effect of increasing concentrations of the anesthetic tetracaine-HCl on the 90° light-scattering (600 nm) of egg lecithin liposomes in phosphate buffer of pH 6.5 and $I = 0.1$. Lipid concentration, $9 \cdot 10^{-4}$ M; $t = 22°C$.

Discussion

In this study, the self-association of tetracaine-HCl in aqueous phosphate buffer solution of pH 6.5 and $I = 0.1$ was followed by three methods: critical micelle concentration values of 0.069, 0.071 and 0.063 M were found by surface tension, solubilization of perylene and enhancement of ANS fluorescence, respectively. The transition from monomers to micelles is known to take place gradually over a narrow range of concentrations rather than abruptly at a discrete concentration [10,18]. Therefore, taking into account that the transition is determined by an empirical, graphical procedure, the differences observed among these results are not surprising, since some variation may be expected for critical micelle concentration values obtained from different experimental methods.

Tetracaine-HCl micelles show a pronounced effect on lipid membranes by reducing almost to zero the light-scattering of phosphatidylcholine liposomes, as illustrated in Fig. 4. This change can be attributed, as already discussed, to liposome damage [22—24]. The sharp reduction in the scattering of the lipid dispersion takes place at an anesthetic concentration roughly similar to its critical micelle concentration. It would appear that tetracaine micelles are able to solubilize lipids and thus perturb the stability of liposomes. A similar action on egg lecithin membranes has been described for conventional detergents such as Triton X-100 [23]. The membrane perturbation induced by tetracaine-HCl takes place at high, nonpharmacological concentrations. However, the effect could be of interest to those investigators engaged in the encapsulation of therapeutic agents by lipid vesicles, since it may be impossible to entrap micelle-forming drugs at concentrations near or above the critical micelle concentration.

Throughout this work the anesthetic was employed as the hydrochloride. Since the aliphatic amine of tetracaine has a pK_a of 8.5 [1] it can be calculated that at the experimental pH of 6.5 the concentration of the protonated form will be approx. 99% and that of the conjugated base 1% [27]. This calculation is valid only when the total anesthetic concentration is below the critical micelle concentration, because the apparent pK_a of cationic acids such as protonated tetracaine decreases on aggregation [28]. Such a behavior may be related to the lowering of apparent pK_a shown by cationic acid indicators upon incorporation to positively charged interfaces, which depends on the interfacial polarity and charge density [29]. Since these parameters are unknown for tetracaine micelles, the magnitude of the pK_a shift cannot be predicted. Therefore, the figure of 1% calculated for the concentration of base at pH 6.5 should be taken as a minimum estimate for the micellized anesthetic: the actual proportion of neutral form will be larger, the lower the pK_a value. As a consequence, tetracaine micelles at pH 6.5 can be considered as mixed aggregates of the cationic form plus some proportion of the neutral form of the anesthetic.

The formation of micelles can be taken as an expression of the tendency of an amphipathic compound to undergo hydrophobic interactions: the smaller the critical micelle concentration the stronger the tendency [8]. The significance of the critical micelle concentration of a certain surfactant is usually

determined by comparison with values tabulated for other related amphiphiles. A search in the literature [18] shows that the critical micelle concentration of tetracaine-HCl is near the value corresponding to decyl trimethylammonium bromide. The critical micelle concentration of this cationic detergent in 0.1 M NaCl solution has been reported to be 0.0595 M. However, neither the polar groups of both compounds nor the experimental conditions of the determinations are identical. Thus, it would be highly speculative to suggest that the hydrophobic moiety of tetracaine-HCl could be compared to a decyl chain. A precise evaluation of the hydrophobicity of the drug would be possible if the critical micelle concentrations of alkyl dimethylammonium halides of different chain lengths in solutions at the same pH used in this work were available.

It is worth mentioning that, by use of a different experimental approach, the hydrophobicity of tetracaine-HCl has been estimated as equivalent to that of an aliphatic chain of 8.5 methylene groups (J. Cerbón, personal communication).

The critical micelle concentration of tetracaine-HCl is well above the concentrations required for the local anesthetic action in nerves [1]. Nevertheless, the finding that this drug is able to form micelles may contribute to a better evaluation of the importance of the hydrophobic binding component in the interaction of pharmacological concentrations of the anesthetic with membranes.

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