ELECTRON SPIN RESONANCE STUDY OF BENZOIC ACID ESTERS, ANALOGS OF LOCAL ANESTHETICS INTERACTION WITH MEMBRANES, AGGREGATION AND HYDROLYSIS

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INTRODUCTION

Magnetic resonance has been widely used to study the interaction between drugs and membranes. The effects of local anesthetics upon membrane structure have been examined making use of NMR, particulary deuterium (1-7), and of spin labels (8-12).

Work from this laboratory has investigated the effect of membrane concentration upon the ionization properties of the local anesthetic tetracaine (TTC), a tertiary amine (Table I) (10,11).

We have also provided spectroscopic evidence for micelle formation by the charged form of the anesthetic and for phospholipid bilayer disruption by this form leading to mixed phospholipid-anesthetic micelles (10,12).

The uncharged form of TTC binds to membranes until reaching saturation, and then a second phase is formed (10,12). These events are modulated by the compromise between two fundamental properties - the partition coefficient and the water solubility of the drug.

We are presently studying the behavior of analogs of tetracaine (Table I). Here we report on the following aspects: 1- change in the ionization constant of the amino group of the analogs upon their binding to membranes; 2- aggregation of their uncharged form; 3- bilayer disruption by the (aggregated) analogs;4- alkaline hydrolysis of the compounds.

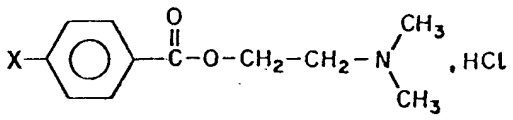
CHANGE IN THE IONIZATION CONSTANT OF THE AMINO GROUP OF THE ANALOGS UPON THEIR BINDING TO MEMBRANES

This study was performed by analysing the ESR spectra of a spin probe (5-MeSL, methyl ester of stearic acid containing the dimethyl-N-oxyl oxazolidine moiety at carbon on 5) incorporated in phospholipid (egg phosphatidyl choline, EPC) bilayers.

Changes in bilayer structure were evaluated by the ratio of the heights of the low (h_{+1}) to the center (h_o) field lines in the spectra of 5-MeSL. The h_{+1}/h_o ratio is an empirical parameter which is affected both by order and by mobility (12-14).

As observed with TTC (1,2,10,11), increasing the pH, there is an increase in the concentration of the uncharged form of the analogs, therefore an increased partitioning of this form into the membrane, leading to a decrease in lipid organization. Plots of the h_{+1}/h_o ratio as a function of pH yield titration curves (Fig. 1) that allow the determination of the apparent pK (pK_{app}) of the analogs. The apparent pK is the aqueous pH at which the total amount of the charged form (in water + membrane) equals the total amount of uncharged form (11). This property depends on membrane concentration (11) and this dependence was verified for the TTC analogs (Fig. 2 and Table II).

TABLE I - Tetracaine and its analogs



X	SYMBOL
$H_3C-CH_2-CH_2-CH_2-NH-$	TTC
CH_3-	CH_3-TTC
H-	H-TTC
Cl-	Cl-TTC

AGGREGATION OF THE UNCHARGED FORM OF TETRACAINE ANALOGS

Figure 3 illustrates aggregate formation. In the absence of analog, a film of the probe (5-MeSL, highly water insoluble) is not solubilized by the aqueous medium and no spectrum is obtained (Fig. 3A). In Fig. 3B the exchange broadened lines indicate a high probe: anesthetic ratio. Since the probe concentration is 1.6×10^{-4} M, this means that only a small fraction of the analog is aggregated at 12 mM. At 38 mM Cl-TTC (Fig. 3C), the lineshape is indicative of a low probe:anesthetic ratio and is similar to that obtained for the probe in micelle-type aggregates (15). The concentrations at which aggregation of the uncharged anesthetics was first detected were: 12 mM (Cl-TTC), 32 mM (CH_3-TTC) and 90 mM (H-TTC).

In contrast to what is observed for charged TTC (10,12), the charged forms of the analogs did not give rise to aggregates at concentrations up to 100, 300, and 120 mM for Cl-TTC, CH_3-TTC, and H-TTC, respectively.

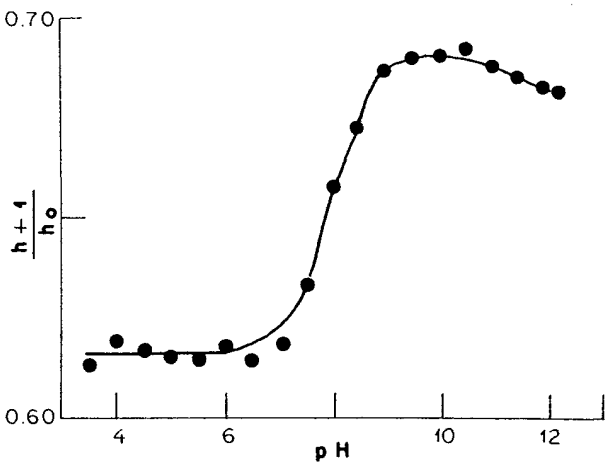


Figure 1: Effect of CH_3-TTC on membrane organization as a function of pH. 50 mM EPC, 40 mM CH_3-TTC, 0.12 M phosphate-borate-citrate (PBC) buffer. The mid-point in the curve yields the apparent pK of the analog. In the absence of the analogs the h_{+1}/h_o ratio is invariant with pH.

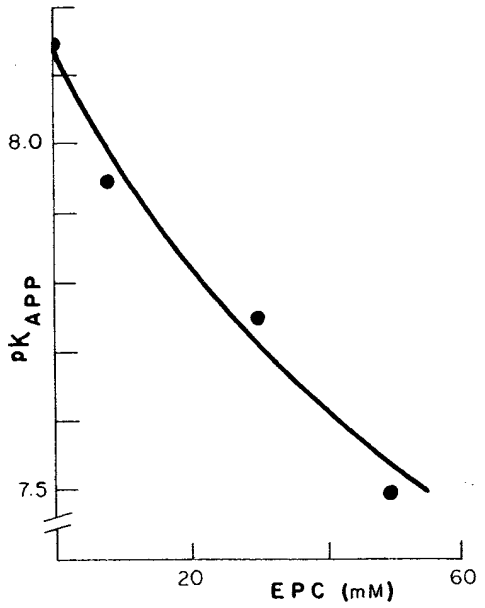


Figure 2: pK_{app} for Cl-TTC as a function of membrane concentration.

Table II - Effect of membrane concentration on pK_{app} of TTC analogs

EPC	pK_{app}			
(mM)	H-TTC	Cl-TTC	CH_3-TTC	TTC*
0		8.15		8.50
3.0				8.14
5.5				7.76
7.8	8.45	7.95	8.35	
10.9				7.55
30.0	8.30	7.75	8.05	
50.0	8.35	7.50	7.90	

* Taken from ref. 11

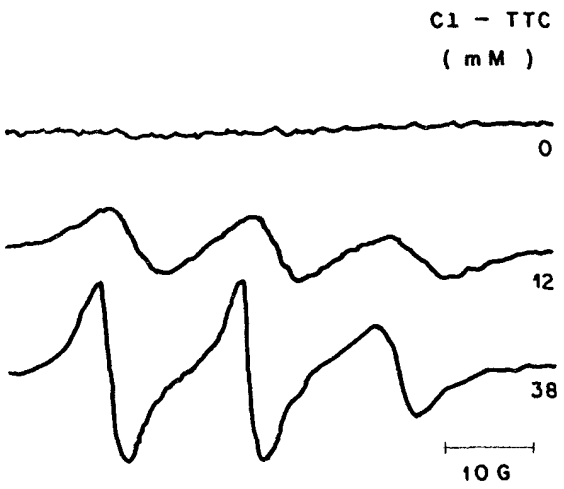


Figure 3: ESR spectra of 5-MeSL in 0.12M PBC buffer, pH 10.5, containing 0 (A), 12 (B) and 38 mM (C) Cl-TTC.

BILAYER DISRUPTION BY THE (AGGREGATED) ANALOGS

When the uncharged form of the TTC analogs is added to EPC bilayers, their partitioning into the membrane gives rise to a less organized system. Figure 4 shows increasing h_{+1}/h_o values with increasing anesthetic concentration, until a breaks occurs. In the concentration range where the break is observed, the system changes from a turbid to an optically clear one, indicating a decrease in particle size. The latter particles probably consist of micelle-like mixed uncharged anesthetic-phospholipid aggregates, generated as a consequence of the tendency of the uncharged form of the analogs to aggregate. Again, these results are in contrast with those observed with TTC, where the charged form is responsible for analogous events (10,12).

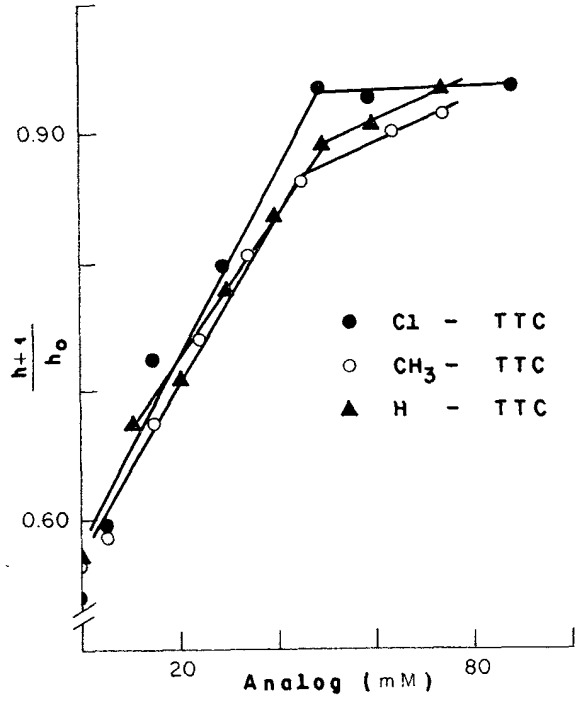


Figure 4: h_{+1}/h_o ratio as a function of TTC analog concentration added to 7.8 mM EPC multibilayers in 0.12 M PBC buffer, pH 10.5. The concentrations of H-TTC are twice those given in the abcissa.

ALKALINE HYDROLYSIS OF TETRACAINE ANALOGS

The analogs of TTC undergo alkaline hydrolysis with the half times given in Table III, as monitored by spectrophotometry. The half times vary as expected from the substituent effect. We found that this process could also be monitored by following the changes occurring in the spectra of spin probes incorporated in the anesthetics aggregates. Figure 5 shows the spectra of a spin probe (5-SASL) in Cl-TTC aggregates as a function of time. The spectrum due to the aggregated structure disappears while a component due to freely tumbling probe appears (arrows). We ascribe the changes to hydrolysis of the ester moiety of the compounds leading to substituted benzoic acid anions and free alcohols. It can also be concluded that the esters, and not the reaction products (which are much more water soluble), are responsible for aggregate formation.

When the kinetics of hydrolysis was studied for anesthetics concentrations in the range where their aggregates start to yield spectra similar to those in Fig. 3C (micelle-like, 38 mM for Cl-TTC, 50 mM for CH_3-TTC and 120 mM for H-TTC), the time scale of spectral changes was in good agreement with the half times in Table III. Increasing the anesthetics concentration led to slower rates of spectral changes, i.e., slower rates of hydrolysis, indicating that aggregation plays a role in the reaction kinetics. This is analogous to what is observed in membranes, where the compounds partitioned into these structures also undergo hydrolysis, but at a slower rate than in aqueous medium (M.L. Bianconi, A.T. do Amaral and S. Schreier, in preparation).

TABLE III - Half times for alkaline hydrolysis of TTC analogs in aqueous medium, pH 10.5

ANALOG	TEMPERATURE (°C)	$t_{1/2}$ (min)
Cl-TTC	25	300
	30	250
H-TTC	25	700
	30	520
CH -TTC	25	1300
	30	1400

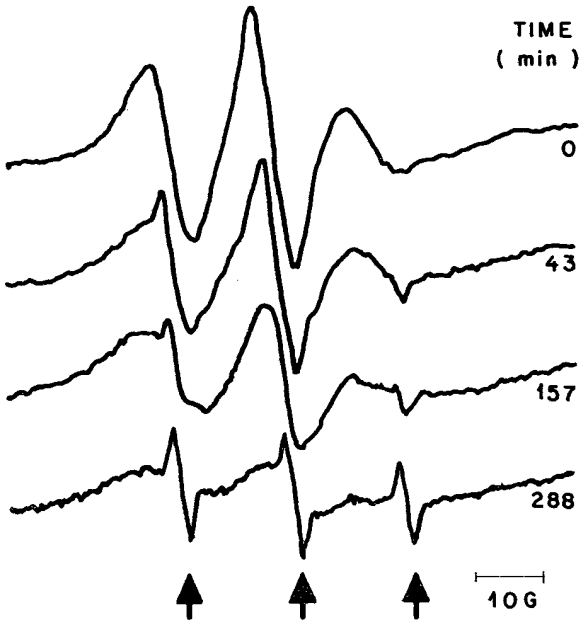


Figure 5: ESR spectra of 5-SASL (stearic acid containing the dimethyl-N-oxyl oxazolidine moiety at carbon 5) incorporated in 30 mM Cl-TTC aggregates as a function of time. 0.12 M PBC buffer, pH 10.5, 25 ± 2 °C.

DISCUSSION

The mechanism of action of local anesthetics is related to their binding to membranes (16). Not only membrane structure is affected, but also, conversely, fundamental physico-chemical properties of the drugs are modified as a consequence of the interaction.

We have designed experiments to study these effects by means of spin label ESR spectra. Thus, we have shown that membranes affect the ionization of the tertiary amino group of TTC (10,11) as well as the rate of hydrolysis of the ester moiety of its analogs (M.L. Bianconi, A.T. do Amaral and S. Schreier, in preparation). Aggregation has also been shown to play a role in the interaction between TTC and membranes (10,12,17,18).

The present work extends our observations and reports on the effect of membrane concentration on the apparent pK of TTC analogs (Fig. 1, Fig. 2 and Table II).

The effect of membrane concentration upon the degree of ionization of a compond that undergoes partitioning is a relevant phenomenon, especially when dealing with pharmacologically active ionizable drugs of which only one form is active.

We also found that the TTC analogs aggregate (Fig. 3) and that they disrupt bilayer membranes (Fig. 4). In contrast with TTC, these events are observed for the uncharged form of the analogs. The effective membrane concentrations of the compounds could not be evaluated since their partition coefficients are not known at present. Experiments to determine these values by ESR are under way.

The last part of this work shows that the hydrolysis of the compounds can be detected by ESR (Fig. 5) provided they are aggregated at the beginning of the reaction. These experiments demonstrate that the kinetics of a chemical reaction can be monitored by the changes in the ESR spectra of a spin probe incorporated in aggregates of the reagent.

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