INTERACTION OF A FLUORESCENT PROCAINE ANALOGUE WITH PHOSPHATIDYLCHOLINE VESICLES

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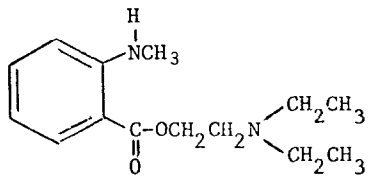
1. Introduction

Although the effects of amine local anesthetics on electrical impulse transmission are well documented, the nature of the molecular interactions between these compounds and the cell membrane is still unclear [1,2]. Association of local anesthetics with lipid bilayers is believed to play at least some role particularly since many physical properties such as phase behavior and fluidity appear to be sensitive to anesthetics [3,4]. On the other hand specific interactions with membrane receptors involved in excitation have also been suggested [5].

Techniques such as NMR, ESR and fluorescence spectroscopy have been used to study the mechanism and site of interaction of local anesthetics with artificial membranes [6–8]. With the exception of recent NMR studies on deuterated procaine [9], those methods often rely on the perturbation of the environment by the anesthetic as detected by incorporated spin or fluorescent probes. Although such well-studied local anesthetics as procaine and tetracaine are weakly fluorescent, their spectral properties are not suitable to allow their use directly as fluorescent probes particularly in natural membranes where spectral overlap between these molecules and protein tryptophans makes unambiguous interpretation exceedingly difficult. Nevertheless, it would be of great value to be able to directly measure the association of anesthetic molecules with biological membranes and to probe the nature of the interaction with both lipid and protein domains.

We report here the synthesis and spectral properties of a fluorescent procaine analogue, *N,N*-diethylaminoethyl(2-*N*-methyl)benzoate–HCl (I) (DANB). Studies on the binding of this molecule with phospholipid vesicles suggest that DANB can be an extremely useful probe to mimic anesthetic interactions with bilayers.



DANB: *N,N*-diethylaminoethyl(2-*N*-methyl)benzoate–HCl (I)

2. Experimental

N,N-Diethylaminoethyl(2-*N*-methyl)benzoate–HCl was synthesized by the general method in [10]. *N*-methylisatoic anhydride (Molecular Probes) (0.75 g) and 0.05 g NaOH was dissolved in 3 ml spectral grade *N,N*-diethylethanolamine and heated for 90 min at 60°C under a positive pressure of N_2. The free amine obtained from this reaction was converted to the hydrochloride salt by acidification of the ethereal solution with concentrated HCl. The salt was recrystallized from hot propanol to give the desired product in 70% yield (m.p. 150.0–150.5°C). Thin-layer chromatography in chloroform–methanol (3:7) indicated a single fluorescent species. Structure and purity was confirmed by IR, ^{1}H-NMR analysis and elemental analysis.

Phospholipid vesicles were prepared by sonicating (Braunsonic, 1510; 10 min at 70 W) 10 mg egg phosphatidylcholine/ml (Sigma) in either H_2O or 15 mM Tris–HCl (pH 7.4). The opalescent solution was centrifuged for 1 h at 100 000 × *g* to remove particulate material.

Fluorescence measurements were made using either

a SLM 4800 spectrofluorometer or a Perkin-Elmer MPF-44B spectrofluorometer equipped with a DCSU corrected spectra unit. Quantum yields were determined using quinine sulfate in 1 N H_2SO_4 as a reference (quantum yield, 0.55 [11]). Samples were excited at 366 nm at an absorbance ranging from 0.01–0.02. All phospholipid samples were equilibrated for 5–10 min at 24°C in the sample compartment prior to measurement. Total fluorescence intensities were obtained by integrating the area under the spectrum from 370–550 nm using a computer spectral analysis program.

3. Results

Fig.1 shows the uncorrected emission and excitation spectra of DANB in H_2O and in 100% ethanol. The emission maximum is blue shifted from 443 nm in H_2O to 423 nm in ethanol with a corresponding enhancement of 4.5-fold in fluorescence intensity.

The emission spectra of DANB in the presence of egg phosphatidylcholine vesicles at pH 7.4 is shown in fig.2. Interaction with vesicles is accompanied by an enhancement of fluorescence intensity as well as a 7 nm blue shift in emission maximum. This is expected if some fraction of the probe were transferred from the polar aqueous environment to a more non-polar lipid domain. Since DANB exists mainly in its protonated form at pH 7.4, its partitioning into the phospholipid bilayer would be relatively low. However, increasing the pH of the solution and therefore the relative concentration of the more non-polar free base, should lead to enhanced binding and a corresponding increase in fluorescence intensity. The pH dependence of the relative intensity (fig.3A) shows that this is indeed the case. In addition, this enhancement is accompanied by a pronounced shift to lower wavelengths (fig.3B). In the absence of phospholipid vesicles, there is no pH-dependent wavelength shift and a considerably smaller change in fluorescence intensity.

A more detailed study of the interaction of the probe with phospholipid vesicles at pH 9.0 is shown in fig.4. Increasing [lipid] from 0.05–3.0 mg/ml leads to a progressive increase in fluorescence intensity and increasing blue shift in wavelength of emission. A plot of wavelength shift as a function of [lipid] at 12.5×10^{-6} M DANB (fig.5) shows that the wavelength reaches a minimum between 3–3.5 mg lipid/ml. In

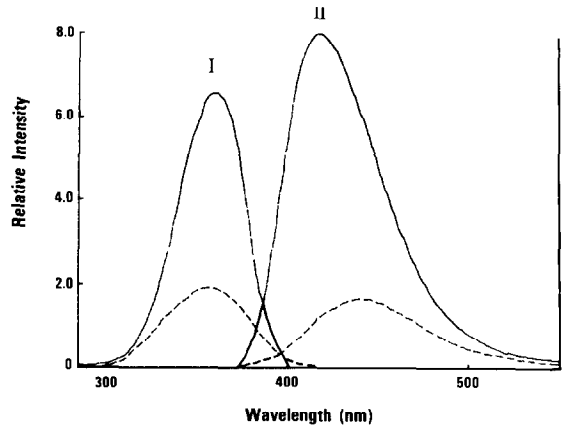


Fig.1. Excitation and emission spectra of DANB. Excitation spectra (I) in H_2O (– –) and ethanol (——) were recorded at an emission wavelength of 445 nm. Corresponding emission spectra (II) were recorded at an excitation wavelength of 350 nm. [DANB] = 5×10^{-6} M.

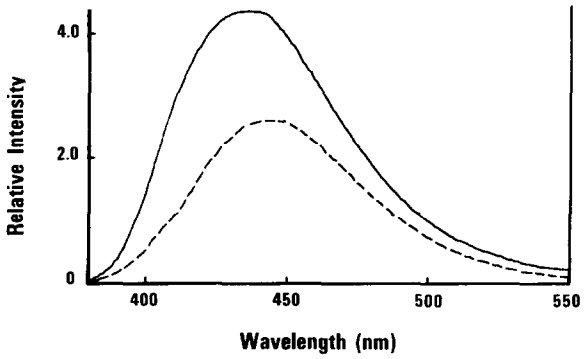


Fig.2. Emission spectra of DANB in the absence (– –) and presence (——) of 3.0 mg phosphatidylcholine/ml in 0.015 M Tris–HCl (pH 7.4). [DANB] = 12.5×10^{-6} M.

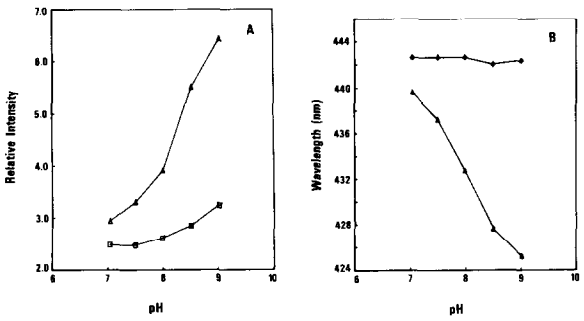


Fig.3. pH dependence of the relative fluorescence intensity (A) and the maximum emission wavelength (B) of DANB in 3.0 mg phosphatidylcholine/ml and 0.015 M Tris–HCl. [DANB] = 12.5×10^{-6} M.

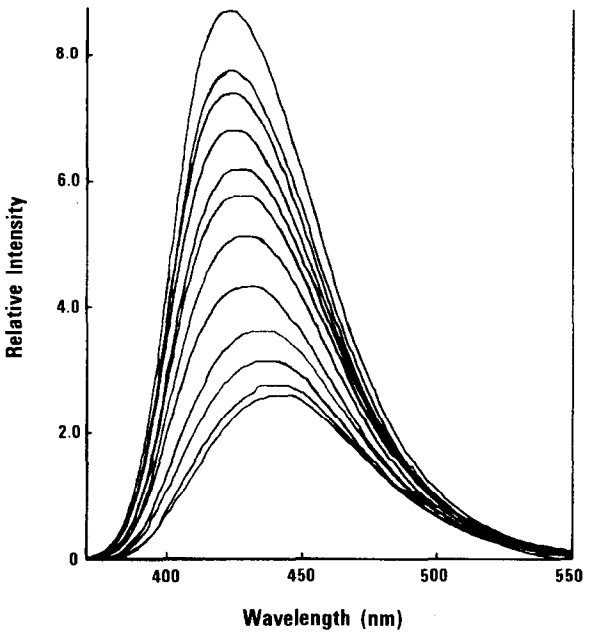


Fig.4. Emission spectra of DANB in phosphatidylcholine vesicles in 0.015 M Tris–HCl (pH 9.0). Phospholipid concentrations in increasing order of intensity were: 0.00; 0.05, 0.10, 0.20, 0.40, 0.60, 0.80, 1.00, 1.25, 2.00, 2.50 and 3.00 mg/ml. [DANB] = 12.5 × 10^{-6} M.

addition, the relative intensity also approaches maximum levels at this lipid concentration (not shown) and one can therefore assume that under these conditions, the probe is totally bound and that the spectral characteristics exhibited at this pH reflect the nature of the binding site.

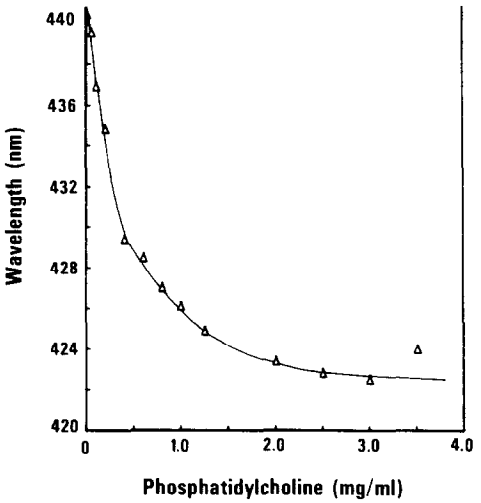


Fig.5. The dependence of the maximum emission wavelength of DANB on the phospholipid concentration. Experimental conditions were as in fig.4.

To determine the environment of the binding site of the probe, the effects of solvent on the emission spectral parameters were studied. As expected, large changes in the fluorescence quantum yield and emission maximum are observed with changes in the ethanol composition of ethanol/H_2O mixtures (table 1). Linear correlations are found between emission maximum and two commonly used polarity scales, one reflecting the dielectric constant of the solvent mixture [12] and the other an empirical polarity scale Z introduced in [13]. The Z polariry scale (fig.6) gave a better fit with a correlation coefficient of 0.995

Table 1
Solvent dependence of the fluorescence properties of DANB

% Ethanol (ethanol/H_2O)	Emission max. (nm)	Quantum yield (ϕ)	Dielectric constant (D)[a]	Z[b]
100	425	0.51	24	79.6
90	429	0.55	29	82.5
80	430	0.56	35	84.8
70	433	0.57	40	86.4
60	436	0.52	47	87.9
50	438	0.49	52	89.2
40	439	0.42	58	90.5
30	441	0.31	64	91.6
20	443	0.22	69	92.6
10	444	0.16	74	93.6
0	445	0.12	80	94.6

[a] From [12]; [b] from [13]

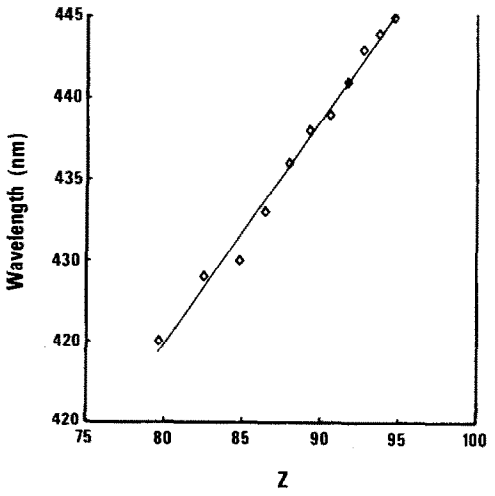


Fig.6. The dependence of the maximum emission wavelength of DANB on the Z value of the solvent in ethanol/H_2O mixtures. [DANB] = 5×10^{-6} M.

compared to 0.987 for the dependence on dielectric constant (not shown). A better correlation between the emission parameters of anilinonapthalene sulfonic acid (ANS) and the Z polarity scale was reported [12]. From fig.5, the limiting emission maximum is 423 nm (uncorrected) or 429 nm (corrected), a value which corresponds to the emission in 90% ethanol and to a Z value of 82 and a dielectric constant of ~29 (table 1). The corresponding value for the fluorescence quantum yield in phospholipid vesicles should therefore be 0.55 compared to 0.12 in H_2O.

An alternative method for determining the quantum yield of fully bound probe involves measuring the extrapolated maximum relative intensity from double reciprocal plots. When the data in fig.4 was expressed in this form plotting reciprocal intensity against reciprocal [lipid], at 12.5×10^{-6} M DANB the extrapolated intercept at the ordinate can be taken to reflect fully bound probe to give a relative quantum yield of 0.42, a value significantly lower than that obtained from the solvent studies. There are several possible explanations for this discrepancy:

(i) Double reciprocal plots tend to underestimate the true maximum fluorescence intensity [14].
(ii) Any quenching of bound fluorophore in the phospholipid vesicles would tend to decrease the estimated quantum yield but should have little effect on the wavelength of emission.

The latter parameter therefore provides a more meaningful measure of the local environment of the probe.

4. Discussion

The use of fluorescent anesthetic derivatives to model the behavior of local amine anesthetics provides a powerful technique to study the mechanism of action of anesthetics at the molecular level. The fluorescent derivative we have described here is a highly suitable probe since it is the *N*-methyl *ortho*-isomer of procaine. The *ortho*-isomers of both procaine and tetracaine have anesthetic activity; the *ortho*-isomer of procaine is even more active than procaine itself [15]. Because of its close structural resemblance to procaine, it is also unlikely to cause anomolous perturbation of the bilayer structure that may occur with other larger heterocyclic aromatic fluorescent probes such as pyrene or anthracene.

The fluorescence characteristics of this derivative also make it highly suitable as a molecular probe. There is minimal overlap between excitation and emission spectra thus allowing one to study probe interactions even in highly scattering solutions. The excitation band is well removed from those of tryptophan and tyrosine residues and perhaps more interestingly, the large overlap between tryptophan emission and DANB excitation bands makes this probe potentially useful in studying energy-transfer phenomena in biological membranes. The high sensitivity of the probe to solvent polarity also allows one to quantitate the binding of the molecule to both natural and artificial membranes and to obtain information on the nature of the binding site(s).

Studies on the fluorescence of DANB in egg phosphatidylcholine vesicles, suggest that the binding environment has a dielectric constant of ~29 and a Z polarity value of 82. These values are within the range of those reported for the polarity of the hydrocarbon–water interface of detergent micelles (Z = 82–85) [16] and of egg phosphatidylcholine bilayers (D = 28) [17], suggesting that the aromatic ring of the anesthetic molecule is situated at or near the lipid–water interface even at pH 9.0.

The physiological effects of amine anesthetics are known to be pH-dependent [18]. Although the charged form of the anesthetic is the biologically active species blocking action potentials from the inner surface of the nerve, it may be the uncharged form that transverses the bilayer [18]. This conclusion is consistent with the observation that the partition coefficient of procaine and tetracaine in phosphatidylcholine/water dispersions increases with increasing pH [9]. Our stud-

ies on the pH dependence of DANB fluorescence in phospholipid bilayers also support this conclusion.

Using deuterium-labelled anesthetics evidence has been provided for multiple binding sites for charged and uncharged forms of local anesthetics [9]. Other studies also suggest polar and non-polar interactions between these molecules and phospholipid bilayers [7]. The steady-state fluorescence spectral characteristics of DANB in egg phosphatidylcholine vesicles can not be interpreted in terms of multiple binding domains although at present this possibility can not be excluded. We are currently attempting to determine the orientation of DANB within the bilayer by the use of localized spin quenchers [19]. These studies should provide valuable insights into the distribution of anesthetics within biological membranes.

Acknowledgement

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References

[1] Strichartz, G. (1976) Anesthesiology 45, 421–441.
[2] Seeman, P. (1972) Pharmacol. Rev. 24, 583–655.
[3] Lee, A. G. (1978) Biochim. Biophys. Acta 514, 95–104.
[4] Papahodjopoulos, D., Jacobson, K., Poste, G. and Shepherd, G. (1975) Biochim. Biophys. Acta 394, 504–519.
[5] Hille, B. (1977) J. Gen. Physiol. 69, 497–515.
[6] Yeagle, P. L., Hutton, W. C. and Martin, R. B. (1977) Biochim. Biophys. Acta 465, 173–178.
[7] Cerbón, J. (1972) Biochim. Biophys. Acta 290, 51–57.
[8] Butler, K. W., Schneider, H. and Smith, I. C. P. (1973) Arch. Biochem. Biophys. 154, 548–554.
[9] Boulanger, Y., Schreier, S., Leitch, L. C. and Smith, I. C. P. (1980) Can. J. Biochem. 58, 986–995.
[10] Staiger, R. P. and Miller, E. B. (1959) J. Org. Chem. 24, 1214–1219.
[11] Melhuish, W. H. (1961) J. Phys. Chem. 65, 229–235.
[12] Turner, D. C. and Brand, L. (1968) Biochemistry 7, 3381–3390.
[13] Kosower, E. M. (1958) J. Am. Chem. Soc. 80, 3253–3260.
[14] Zierler, K. (1977) Biophys. Struct. Mech. 3, 275–289.
[15] Büchi, J. von, Koller, R. J. and Perlia, X. (1975) Arzneim.-Forsch. (Drug Res.) 25, 14–24.
[16] Gilter, C. and Cordes, E. H. (1973) Prog. Bioorg. Chem. 2, 1–53.
[17] Shinitzky, M. (1974) Isr. J. Chem. 12, 879–890.
[18] Narahashi, T., Frazier, D. T. and Yamada, M. (1970) J. Pharmacol. Exp. Ther. 171, 32–44.
[19] Luisetti, J., Möhwald, H. and Galla, H. J. (1979) Biochim. Biophys. Acta 552, 519–530.