Effect of the Local Anesthetic Heptacaine Hydrochloride on the Structured Water in Model Phosphatidylcholine Membrane: ^{2}D-NMR and ^{31}P-NMR Study

J. Národa[1], P. Balgavý[1,2], K. Gawrisch[3] and J. Čižmárik[2]

1 *Institute of Physics and Biophysics, Comenius University, 842 15 Bratislava, Czechoslovakia*
2 *Faculty of Pharmacy, Comenius University, 832 32 Bratislava Czechoslovakia*
3 *Section of Physics, K. Marx University, 7001 Leipzig, GDR*

Abstract. Interaction of the local anesthetic 1 [2-(2-heptyloxyphenyl-carbamoyloxy)-ethyl] piperidinium chloride (heptacaine hydrochloride) with a model membrane formed by phosphatidylcholine was studied using ^{2}D-NMR spectra of heavy water and ^{31}P-NMR proton-decoupled and undecoupled spectra of the lipid phosphate group. Heptacaine hydrochloride was found to increase the number of water molecules oriented over the polar region of the membrane, to increase the order parameter of the lipid phosphate group, as well as that of oriented water, and to increase the volume of unoriented water trapped between the bilayers. Heptacaine hydrochloride also affects the temperature dependence of quadrupolar splitting of oriented heavy water deuterons. Heptacaine hydrochloride is suggested to expand the membrane laterally and to charge the membrane surface electrostatically.

Key words: Local anesthetic — Heptacaine hydrochloride — Structured water — Model membranes — ^{2}D-NMR — ^{31}P-NMR

Introduction

Several theories have been proposed for the mechanism of action of local anesthetics. They have been proposed to act at the membrane level by interacting with proteins or lipids. Theories involving proteins have focused on the interaction with a specific receptor (Hille 1980) or on nonspecific binding to hydrophobic sites (Richards et al. 1980). Studying interaction with lipids, effects on the lipid phase transition temperature, on enthalpy and cooperativity of transition from gel to the liquid crystalline phase (Hill 1974; Jain and Wu 1977; Davio and Low 1981), as well as lateral phase seprations (Trudell 1980; Balgavý and Ondriaš 1982), and rising of new phases (Otero and Otero 1975; Cullis and Verkleij 1979; Cullis et al. 1980; Hornby and Cullis 1981), have been observed.

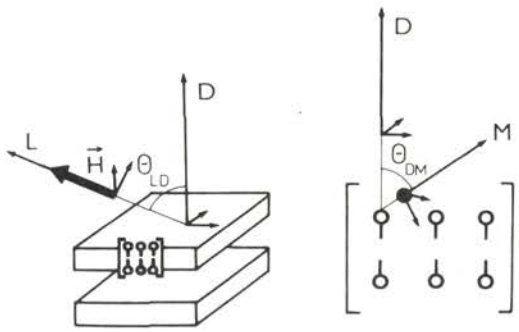

Fig. 1. Schematic drawing of the mesomorphous structure in a lipidic lamellar phase. L — laboratory frame, D — director frame and M — molecular frame. ϑ_{LD} and ϑ_{DM} are the angles between the z-axes of the laboratory — director systems, and the director — molecular systems, respectively. The dark circle represents the oriented water molecule.

Ueda et al. (1980) suggested that the site of action of local anesthetics might be "interfacial", without specifying molecular details of this interaction. In the case of an interfacial action of local anesthetics, a change in structured water should also occur in the vicinity of the membrane surface. The aim of the present paper is to investigate the possibility of a such change using deuterium magnetic resonance spectroscopy (^{2}D-NMR) of heavy water structured over a model phosphatidylcholine membrane, and to study the polar head group conformation and/or mobility in the model membrane using phosphorus magnetic resonance spectroscopy (^{31}P-NMR). The local anesthetic studied in our study is heptacaine, 1[2-(2-heptyloxyphenylcarbamoyloxy)-ethyl] piperidinium chloride; its synthesis and various biological activities have been described earlier (Čižmárik and Borovanský 1975; Čižmárik et al. 1976).

Theory

^{2}D-NMR spectroscopy

In a lipid mesophase, the tumbling of lipid molecules-bound water molecules is anisotropic. Therefore, ^{2}D-NMR spectra of D_2O show a doublet. This characteristic splitting arises from the fact that interaction of the quadrupolar moment, Q, of the nucleus ^{2}D with the electric field gradient tensor of the water molecule, $\vec{\mathbf{V}}$, shifts the energy levels of the Zeeman interaction (Abragam 1961). For the oriented water molecule, using a suitable choice of the axis systems (see Fig. 1), and transformation of the spherical tensors, and considering cylindrically symmetric

water molecule motion around the director, the quadrupolar splitting

$$\Delta = |\nu_Q S(3\cos^2\vartheta_{LD} - 1)| \tag{1}$$

is obtained, where ϑ_{LD} is the angle between the applied magnetic field and the director; $\nu_Q = 3e^2Qq/4h$ is the quadrupolar coupling constant; q is the value of the largest component of the $\vec{\vec{V}}$ tensor; and S is the order parameter (Lindblom et al. 1974). The order parameter is described as

$$S = \frac{1}{2}[(3\cos^2\vartheta_{DM} - 1) + \eta\sin^2\vartheta_{DM}\cos^2\varphi_{DM}] \tag{2}$$

where ϑ_{DM} is the angle between the director and the direction of the largest component of the $\vec{\vec{V}}$ tensor, φ_{DM} is the angle between the intersection line of the XY planes of the director and the molecular axis systems, and the Y axis or the molecular axis system, respectively. $\eta = (V_{XX} - V_{YY})/V_{ZZ}$ is the assymetry parameter, where V_{XX}, V_{YY}, V_{ZZ} are the components of the $\vec{\vec{V}}$ tensor. Quadrupolar splitting, Δ, is determined by both orientation and motion of the various deuterons in the sample, i. e. by the contributions from deuterons bound to different binding sites characterized by the respective intrinsic quadrupolar splittings Δ_i. In the case of a rapid deuteron exchange between the different binding sites, the observed quadrupolar splitting is then given by

$$\Delta = \sum_i p_i\Delta_i \tag{3}$$

where p_i is the probability to find the considered deuteron at the site i, characterized by the intrinsic quadrupolar spliting, Δ_i.

^{31}P-NMR spectroscopy

Since the phosphate group of the lipid molecule moves anisotropically in the liquid crystal mesophase, ^{31}P-NMR spectroscopy can provide information on the structural and/or dynamical properties of the phospholipid mesophases (Seelig 1978; Cullis and Kruijff 1979)). The description of the phosphate group orientation and motion is similar to that used in ^{2}D-NMR spectroscopy for describing the water molecule orientation and motion.

Transforming the ^{31}P chemical shift anisotropy tensor, $\vec{\vec{\sigma}}$, from the molecular to the laboratory frame, and supposing the lipid phosphate group motion around a director with a cylindrical symmetry, the effective chemical shift anisotropy may be written as

$$\Delta\sigma_{eff} = \left|\left(\sigma_{33} - \frac{\sigma_{22} + \sigma_{11}}{2}\right)\frac{1}{2}(3\cos^2\vartheta_{MD} - 1) + \frac{3}{4}(\sigma_{11} - \sigma_{22})\sin\vartheta_{MD}\cos 2\varphi_{MD}\right|, \tag{4}$$

where σ_{11}, σ_{22}, σ_{33} are the components of the diagonalized $\vec{\vec{\sigma}}$ tensor in the moledular axis system; ϑ_{DL} is the angle between the aplied magnetic field and the director, ϑ_{MD} is the angle between the director and σ_{33}, and φ_{MD} is the angle between the intersection line of XY planes of the director and the molecular axis system, and the Y axis of the molecular axis system. In the case of a rapid motion, the observed chemical shift anisotropy is given by

$$\Delta\sigma_{eff} = \sum_i P_i \Delta\sigma^i_{eff} \tag{5}$$

where P_i is the probability to find the considered nucleus at the site i, characterized by $\Delta\sigma^i_{eff}$.

The position of the isotropic ^{31}P-NMR signal is given by

$$\sigma_{iso} = \frac{1}{3}(\sigma_{11} + \sigma_{22} + \sigma_{33}). \tag{6}$$

Material and Methods

Egg yolk lecithin (EYL) was isolated and purified according to Singleton et al. (1965). Heptacaine was synthesized as described earlier (Čižmárik and Borovanský 1975). The solvents used were of p. a. grade. Heavy water (D_2O), isotopic purity 99.5% (obtained from ÚVVVR, Prague, Czechoslovakia) was used without any further purification.

Samples were prepared as follows. EYL was vacuum-dried (10^{-2} Pa) for 18 hours, dissolved in chloroform; heptacaine was then added to this solution. The EYL-heptacaine solution was evaporated and vacuum-dried (10^{-2} Pa) for 18 hours, and subsequently transferred to glass tubes. D_2O was added with a microsyringe, and the tubes were sealed. The samples were homogenized by centrifugation at room temperature, and by repeated freezing and thawing. The error of the water concentrations was ± 6 %.

Spectra were recorded on a NMR spectrometer Bruker HX-90 with the Fourier transformation. Typical spectrometer parameters for ^{2}D-NMR and ^{31}P-NMR spectra recordings were: frequency 6.492 MHz and 36.43 MHz, respectively, pulse width 20 μs and 8 μs, respectively, pulse distance 12 ms and 700 ms, respectively. Ouadrupolar splitting was determined with an accuracy of ± 20 Hz, the effective chemical shift anisotropy determination accuracy was ± 2 ppm. The temperature accuracy was ± 1 K. Some of the ^{31}P-NMR spectra were recorded under strong-gated proton-noise decoupling using a home-built 600 W unit.

Results

Typical ^{2}D-NMR and ^{31}P-NMR proton decoupled spectra obtained from EYL-D_2O-heptacaine hydrochloride systems are shown in Figs. 2 and 3. The ^{31}P-NMR spectra obtained are characteristic of the lamellar phase L_α (Seelig 1978; Cullis and Kruijff 1979). The nature of the ^{2}D-NMR spectra varied with the water concentration. In samples without heptacaine a doublet was observed after

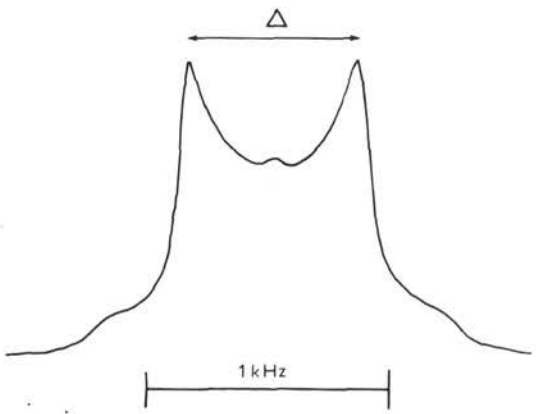

Fig. 2. Typical ^{2}D-NMR spectrum of the EYL-D_2O-heptacaine hydrochloride system. Molar ratios: EYL: heptacaine hydrochloride = 2.9:1 and EYL: D_2O = 1:23. Δ is the quadrupolar splitting.

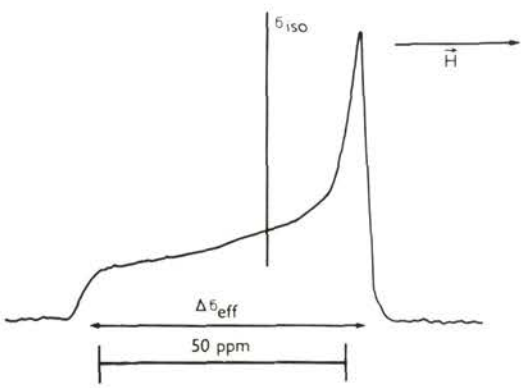

Fig. 3. Typical ^{31}P-NMR spectrum of the EYL-D_2O-heptacaine hydrochloride system. Molar ratios EYL: heptacaine hydrochloride = 2.9:1 and EYL: D_2O = 1:23. σ_{iso} and $\Delta\sigma_{eff}$ are described in the Theory section and they are exposed in ppm. $\vec{H}$ shows the direction of the magnetic field.

equilibration in the spectra at low water concentrations. At D_2O:EYL molar ratios exceeding 23, a singlet corresponding to isotropically tumbling water molecules was superimposed on the doublet. This spectral shape does not change with the time after equilibration. No significant changes in the shapes of the spectra occurred after a 6-months storage at -20 °C in a refrigerator; the amplitude of the singlet increased with the increasing D_2O: EYL molar ratio.

In contrast to drug free systems, those containing heptacaine hydrochloride, showed in ^{2}D-NMR spectra a central peak corresponding to isotropically tumbling water molecules, also at water concentrations below 23 moles of water per one

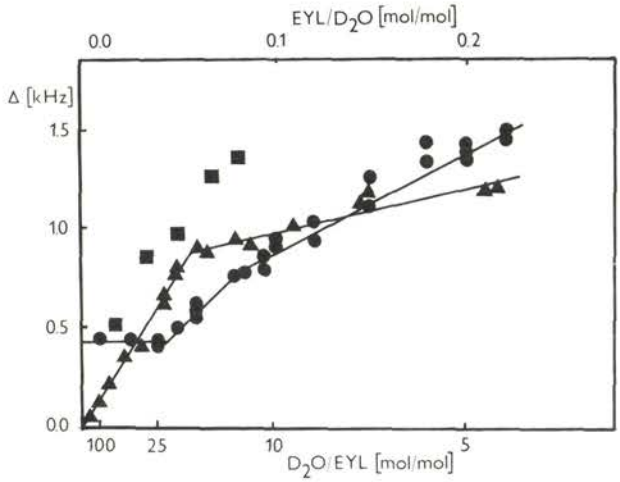

Fig. 4. Dependence of the quadrupolar splitting on the D_2O:EYL molar ratio. (circles) — heptacaine hydrochloride free systems, (triangles) and (squares) — heptacaine hydrochloride-containing systems with EYL: heptacaine hydrochloride molar ratios of 2.9:1 and 0.96:1, respectively.

mole of lipid. However, this central peak in ^{2}D-NMR spectra disappeared upon incubation; this may indicate equilibration of samples. Only one doublet occurred in the spectra of heptacaine-containing samples after equilibration, regardless of their water concentration.

The quadrupolar splitting, Δ, obtained from the doublet splitting is shown as a function of the D_2O:EYL molar ratio (Fig. 4). It can be seen that the plots consist of several straight line areas. The addition of heptacaine induced changes in the widths of the straight line areas, characterized by different line slopes, as well as changes in the line slopes of these areas. Control samples with no heptacaine hydrochloride added showed three straight line areas. Starting from the water poor region, these lines intersect the vertical axis of the plot (Fig. 4) at 0.38 kHz; 0.0 kHz; and 0.43 kHz. The EYL:D_2O molar ratios at which the slopes are changing were 1:12.5 and 1:23.0. Ternary systems with a EYL: heptacaine hydrochloride molar ratio of 2.9:1 showed only two straight line areas. The intersection points were 0.76 kHz and -0.04 kHZ, and slope change was observed at a EYL:D_2O molar ratio of 1:17.9. At a EYL: heptacaine hydrochloride molar ratio of 0.96, the number of experimental points was insufficient to reflect any changes except for an increase in quadrupolar splitting.

The quadrupolar splitting in ^{2}D-NMR spectra of EYL-D_2O-heptacaine hydrochloride systems increased with the increasing temperature, as shown in Fig. 5. We have tried to fit experimental data to the $\ln \Delta \sim 1/T$ linear dependence by the

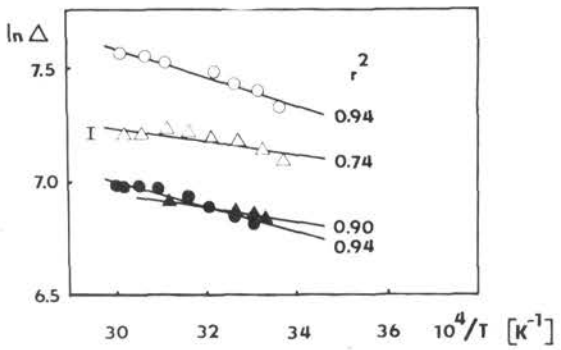

Fig. 5. Temperature dependence of quadrupolar splitting Δ (in Hz) for EYL-D_2O systems with EYL: D_2O molar ratios of 1 : 4.5 (empty circles) and 1 : 10.5 (full circles); and for EYL-D_2O-heptacaine systems with EYL: D_2O of molar ratios 1 : 6.7 (empty triangles) and 1 : 15.4 (full triangles), and EYL: heptacaine hydrochloride = 2.9 : 1. r^2 is the coefficient of determination in the least-squares method.

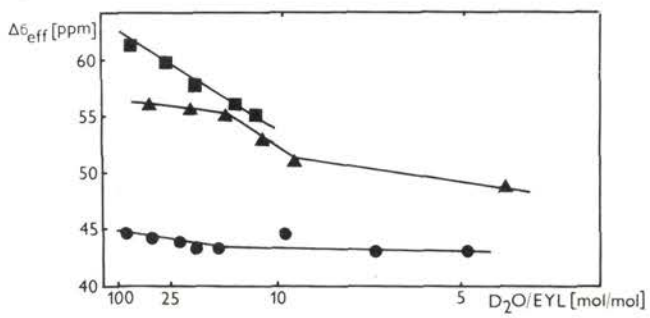

Fig. 6. Dependence of the effective chemical shift anisotropy on the D_2O : EYL molar ratio. (circles) — heptacaine hydrochloride free system, (triangles) and (squares) — heptacaine hydrochloride containing systems with EYL : heptacaine hydrochloride molar ratios of 2.9 : 1 and 0.96 : 1, respectively.

method of the least-squares. This fitting was satisfactory in the terms of the coefficient of determination r^2, as shown in Fig. 5, except for the sample with heptacaine and a lower water concentration. In the latter case, the value of r^2 was affected by two points obtained at high temperatures, where the experimental error (indicated by a bar) was too high. The presence of heptacaine hydrochloride did not affect the typical „lamellar" shape of ^{31}P-NMR proton-decoupled spectra of the systems examined. The effective anisotropy of the ^{31}P-NMR chemical shift, $\Delta\sigma_{eff}$, calculated from the spectra, increased with the increasing heptacaine hydrochloride concentration as well as with increasing water concentration as shown in Fig. 6.

according to Cowley et al. (1978)). Rand et al. (1980) using X-ray diffraction found $d_w = 1.9$ nm for synthetic dipalmitoylecithin in excess water. Since the distance between the lamellae, d_w, is a function of the bilayer repulsion (hydration) force, the equilibrium distance, $d_w = \text{const.}$ indicates the formation of the bulk water as a separate phase. Since the quadrupolar splitting in the main hydration shell increases with the decreasing n (see Fig. 4), the main hydration shell probably consists of different "subshells". One of us (Gawrisch et al. 1978) proposed a model, where the first 5 water molecules per lipid interact strongly with the phosphate group and the other water molecules of the main hydration shell are attached to the lipid head group rather firmly. This model was further supported by computer experiments using Monte Carlo and molecular dynamics calculations (Frischleder and Peinel 1982) and by the Raman spectra analysis which indicates that the first four to five water molecules of water induce a rotation of the phosphate group around the bilayer normal (Mushayakara et al. 1982). Unfortunatelly, the scatter in experimental data at $n < 6$ (see Fig. 4) hindered us to examine them in this region using this model.

Heptacaine affects the properties of water in the model membrane system in several respects. After equilibration the main feature of all the spectra of samples containing heptacaine was the disappearance of the singlet typical for the free bulk water, and a decrease in splitting with the increasing water, content. As seen in Fig. 4, this decrease is a linear function of n^{-1} for $200 > n > 18$ within the experimental error; the intrinsic splitting for this region is very small (-40 Hz) and indicates an isotropic motion. In analogy with the heptacaine-free system, this kind of water can thus be described as trapped water. Using the concept of the equilibrium distance developed above, we can calculate the distance between the bilayers from the volume of the trapped water. In the presence of the anesthetic, the volume of the trapped water reached at least 200 water molecules per lipid corresponding to the equilibrium distance $d_w = 17$ nm, using the value of $S_c = 0.72$ nm^2 per lipid in excess water according to Cowley et al. (1978). Since at anesthetic: lipid molar ratio of 1:10 approximately 4% lateral expansion of the membrane occurs as described below, the calculated value of the equilibrium distance of expanded membranes will probably be lower. Nevertheless, taking into account that the membrane expansion at an anesthetic: lipid molar ratio of 1:3 results in an increase of the cross-sectional area by 12% ($S_c = 0.80$ nm^2), a very high value of $d_w > 15$ nm is obtained again. These data show that the local anesthetic heptacaine hydrochloride increases the equilibrium distance between the bilayers at least to its sevenfold. What is the cause of this increase? Because of the high heptacaine hydrochloride partition coefficient between the hydrophobic solvent and water (Pešák et al. 1980), at water concentrations used in our experiments all the heptacaine molecules are in fact incorporated into the membrane. Heptacaine is positively charged so that its inclusion into the membrane results in an increase of the surface charge of the

bilayer. Because of the electrostatic repulsion of the charged bilayers, the equilibrium distance becomes enlarged and the volume of trapped water increases while the volume of the bulk water becomes decreased.

Results presented in Fig. 4 indicate further that heptacaine also affects the main hydration shell — the number of oriented water molecules increases up to approx. 18 per lipid, and the intrinsic splitting of the oriented water increases from 0.38 kHz to 0.76 kHz. The increase in the number of oriented water molecules could be explainted by two hypothetical processes:

The insertion of the heptacaine molecule into the membrane orients polar groups of the anesthetic with respect to the director axis system. It may be expected that both the quaternary nitrogen and carbonyl oxygen of the anesthetic are able to hydrate and to orient water molecules as well. The increased number of oriented water molecules might thus be ascribed to the hydration properties of the oriented molecule of the anesthetic. Recalculating the data from Fig. 4, 12 oriented water molecules per one amphiphilic molecule in the membrane (EYL or anesthetic) are obtained; this result is identical with that obtained with the anesthetic-free system.

The second possible process resulting in an increase in the number of oriented water molecules might be the lateral expansion of the membrane caused by the local anesthetic. In the case of tetracaine, Boulanger et al. (1981) have observed an enlargement of the phosphatidylcholine cross-sectional area of about 4% at an anesthetic: lipid ratio of 1:10. Lateral expansion of the membrane could create new adsorption sites for water and thus increase the number of oriented water molecules. Some support for this possibility are the observations of Jendrasiak and Mendible (1976). On the basis of adsorption isotherms, they found that the amount of water adsorbed to the membrane increased with the increasing lipid cross-sectional area in the case of phosphatidylcholines with different hydrocarbon chains.

A further effect observed in our experiments was an increase in the intrinsic quadrupolar splitting of the main hydration shell. As follows from equation (1), this observation indicates a change in the order parameter of oriented water. As the order parameter in (2) includes both the motional and orientational contributions, its changes may be caused by changes in the motion and/or by changes in the lipid head group conformation. However, the first possibility is ruled out by the ^{31}P-NMR data. An increase in the ^{31}P chemical shift anisotropy (Fig. 6) as well as a change in the shapes of ^{31}P-NMR undecoupled spectra (Fig. 7) were observed in the presence of heptacaine hydrochloride. The shapes of undecoupled spectra are affected mainly by the proton-phosphorus dipolar coupling, which is dependent on the head group conformation, and not on parameters of motion. We have calculated several undecoupled spectra with different values of proton-phosphorus dipolar coupling constants, $\bar{\alpha}_A$ and $\bar{\alpha}_B$ (see Fig. 8) using the theory developed by Arnold et al. (1979a, b). Comparison of the spectra in Fig. 8 and Fig. 7 indicates changes in $\bar{\alpha}_A$ and $\bar{\alpha}_B$ values resulting from the action of the anesthetic. The