A Study on the Interaction of Local Anesthetics with Phospholipid Model Membranes by Infrared ATR Spectroscopy§

M. Schöpflin,*† U. P. Fringeli,*†‡ and X. Perlia†

Contribution from the Institute of Pharmacy, Swiss Federal Institute of Technology, CH-8092 Zurich, Switzerland, and the Institute of Physical Chemistry, University of Vienna, A-1090 Vienna, Austria. *Received September 12, 1986*

Abstract: The mechanism of local anesthetic action has been investigated in situ by infrared attenuated total reflection spectroscopy. The lipid bilayer model membrane consisted of a dipalmitoylphosphatidic acid monolayer attached with the polar head groups to the ATR plate and a 1-palmitoyl-2-palmitoleoyl-*sn*-glycero-3-phosphocholine (POPC) monolayer exposing the polar head group toward the aqueous phase. Local anesthetics (procaine, oxybuprocaine, falicaine, cinchocaine) were dissolved in ^2H_2O buffer (0.1 M sodium chloride, 0.02 M phosphate, pH 7.0) at concentrations between 0.001 and 0.75 M. IR-ATR spectra were scanned at equilibrium which was reached at latest 10 min after the contact of the solution with the membrane was established. IR spectra revealed unambiguously that all local anesthetics investigated in this work tend to adsorb in multilayers to the membrane already at pharmacologically relevant concentrations. This observation strongly supports an unspecific mechanism of local anesthetic action by sealing the membrane, i.e., blocking the trans membrane signal transfer. Adsorption and desorption were found to be reversible processes depending only on the local anesthetic concentration of the membrane contacting solution. Experimental adsorption isotherms were analyzed in the framework of the BET model. Two parameters of the BET isotherm were found to be relevant for a quantitative description of local anesthetic activity. The first one (b) is predominantly dependent on the difference of the adsorption enthalpies of the first layer and of consecutive layers, respectively. The higher b the greater is the tendency of the adsorbate to grow laterally, i.e., to seal the membrane. The second parameter (c_0) indicates the concentration at which membrane coverage is completed. Therefore a local anesthetic should exhibit a large b and a small c_0 value in order to be efficient. It may be concluded that in situ IR-ATR measurements are generally applicable for drug screening and therefore can be of significant help for the reduction of experiments with living animals.

The site of local anesthetic action is generally accepted to be at the nerve cell membrane. The molecular mechanism, however, is still the subject of speculations and hypotheses. The interaction between local anesthetics and the phospholipids of the nerve cell membrane was often discussed as a possible basis of local anesthetic action.

The methods used to study the influence of local anesthetics on lipids are manifold: Investigations were made by use of film balance,[1,2] by measuring the conductance of lipid membranes,[3,4] and by measuring the change of the phase transition temperature of lipids.[5,6] NMR spectroscopic studies resulted in information on a molecular level.[7-12]

The main point of interest concerned the localization of the local anesthetics in the membrane, the kind of binding to the lipid molecules, and the degree of specification of the anesthetic interaction with the components of the membrane, i.e., predominantly with lipids and proteins.

Several conceptions of local anesthesia were advanced based on these results, such as change of the physical properties of the nerve membrane through a penetration of the molecules between the lipid molecules,[1] a structural perturbation of the membrane proteins,[13] or closing of the sodium channels through decreasing of the rigidity of the surrounding lipid microenvironment.[14] NMR studies resulted in a localization of local anesthetic molecules in a stretched state between the phospholipid molecules and parallel to the fatty acyl chains.[7-10] More recently Kelusky et al.[11] have proposed a two site model, where the local anesthetic in solution is in fast exchange between sites weakly bound to the membrane and in slow exchange with strongly bound sites in the membrane. A mechanism postulated by Büchi[15] suggests a mechanical sealing of the membrane by adsorption of local anesthetic molecules to the membrane surface. It was based on studies of surface activity, pK_a, adsorbance to surfaces, and changing of the surface tension of lipid films, cf. also Büchi and Perlia.[16,17] In order to get a better molecular understanding of the influence of local anesthetics, such as procaine, oxybuprocaine, falicaine, and cinchocaine, on lipid model membranes consisting of 1-palmitoyl-2-palmitoleoyl-*sn*-glycero-3-phosphocholine (POPC) which is a common lipid in the nerve cell membranes we have applied IR-ATR spectroscopy (ATR = attenuated total reflection). This method is a very suitable tool to study the molecular structure of biomembranes as well as the interaction with pharmacologically active substances. In contrast to most other experimental techniques IR-ATR spectroscopy enables in situ studies of biomembranes on a molecular level and under rather realistic conditions. For details of the application to biological systems see Fringeli et al.[18-20]

Theory

1. ATR Spectroscopy. Integrated Molar Absorption Coefficient and Surface Concentration. For a general review on quantitative

† Swiss Federal Institute of Technology.
‡ University of Vienna.
§ In memorium to J. Büchi.

(1) Skou, J. C. *Acta Pharmacol. Toxicol.* **1954**, *10*, 317.
(2) Büchi, J.; Perlia, X.; Tinani, M. *Arzneim.-Forsch.* **1971**, *21*, 2074.
(3) Büchi, J.; Müller, K.; Perlia, X. *Arzneim.-Forsch.* **1966**, *16*, 1475.
(4) McLaughlin, S. *Molecular Mechanism of Anesthesia, Progress in Anesthesiology*, Fink, B. R., Ed.; Raven Press: New York, 1975; Vol. 1, p 193.
(5) Papahadjopoulos, D. *Biochim. Biophys. Acta* **1975**, *394*, 504.
(6) Lee, A. G. *Biochim. Biophys. Acta* **1978**, *514*, 95.
(7) Boulanger, Y.; Schreier, S.; Leitch, L. C. *Can. J. Biochem.* **1980**, *58*, 992.
(8) Boulanger, Y.; Schreier, S.; Smith, I. C. P. *Biochemistry* **1981**, *20*, 6824.
(9) Kelusky, E. C.; Smith, I. C. P. *Biochemistry* **1983**, *22*, 6011.
(10) Kelusky, E. C.; Smith, I. C. P. *Mol. Pharmacol.* **1984**, *26*, 314.
(11) Kelusky, E. C.; Boulanger, Y.; Schreier, S.; Smith, I. C. P. *Biochim. Biophys. Acta* **1986**, *856*, 85.
(12) Schlieper, P.; Michaelis, L. *Biophys. Struct. Mech.* **1983**, *10*, 1.
(13) Seeman, P. *Pharmacol. Rev.* **1972**, *24*, 583.
(14) Lee, A. G. *Anesthesiology* **1979**, *51*, 64.
(15) Büchi, J. *Pharm. Acta Helv.* **1967**, *42*, 534.
(16) Büchi, J.; Perlia, X. *Beziehungen zwischen den physikalisch-chemischen Eigenschaften und der Wirkung von Lokalanästhestica*; Editio Cantor: Aulendorf, 1962.
(17) Büchi, J.; Perlia, X. *Beziehungen zwischen den physikalisch-chemischen Eigenschaften und der Wirkung von Lokalanänesthetica*; 2. Teil, ETH Zurich, 1976.
(18) Fringeli, U. P. *Z. Naturforsch., C: Biosci.* **1977**, *32C*, 20.
(19) Fringeli, U. P.; Günthard, Hs. H. *Infrared Membrane Spectroscopy*, In *Molecular Biology, Biochemistry and Physics*; Grell, E., Ed.; Springer Verlag: Berlin, Heidelberg, 1981, Vol. 31, p 270.
(20) Fringeli, U. P.; Leutert, P.; Thurnhofer, H.; Fringeli, M.; Burger, M. M. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1315.

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ATR spectroscopy the reader is referred to Fringeli.[21]

The molar absorption coefficient $\epsilon(\tilde{\nu})$ (extinction coefficient [cm²/mol]) is defined according to Lambert–Beer's law

$$T_{\parallel,\perp}(\tilde{\nu}) = \exp[-R\epsilon(\tilde{\nu})cd_{e\parallel,\perp}(\tilde{\nu})] = 10^{-OD_{\parallel,\perp}(\tilde{\nu})} \quad (1)$$

The meaning of the symbols is as follows: T, transmission; $\parallel,\perp$, parallel and perpendicular polarized incident light; $\tilde{\nu}$, wavenumber [cm⁻¹]; R, number of active internal reflections; c, concentration [mol/cm³]; d_e, effective thickness [cm]; OD, optical density. The effective thickness $d_e(\tilde{\nu})$ was introduced in order to facilitate quantitative comparison of ATR spectra with transmission spectra.[22]

In this paper the integrated molar absorption coefficient A was used as a relevant parameter of the band intensity

$$A = \int_{band} \epsilon(\tilde{\nu})\, d\tilde{\nu} = \frac{\ln 10}{Rcd^{iso}_{e\parallel,\perp}} \int_{band} OD^{iso}_{\parallel,\perp}(\tilde{\nu})\, d\tilde{\nu} \quad (2)$$

where $d^{iso}_{e\parallel,\perp}$ denotes the effective thickness of an isotropic bulk sample when parallel and perpendicular polarized incident light is used.

Experimentally, the slopes $k_\parallel$ and $k_\perp$ of the $\int_{band} OD^{iso}_{\parallel,\perp}(\tilde{\nu})\, d\tilde{\nu}$ vs. c plots are determined. Thus A can be calculated according to

$$A = \frac{k_\parallel \ln 10}{Rd^{iso}_{e\parallel}} = \frac{2n_1(\cos\theta)k_\parallel}{Rn_2d_p(E_x^2 + E_z^2)} \ln 10 \quad (3)$$

and

$$A = \frac{k_\perp \ln 10}{Rd^{iso}_{e\perp}} = \frac{2n_1(\cos\theta)k_\perp}{Rn_2d_pE_y^2} \ln 10 \quad (4)$$

where n_1 and n_2 denote the refractive indices of the ATR plate and the sample, respectively, θ is the angle of incidence, E_x, E_y, and E_z are the components of the electric field in the rarer medium, and d_p denotes the penetration depth of the internally reflected light into the rarer medium (sample).

Introducing the integrated molar absorption coefficient A in the equations described earlier[23] one obtains for the surface concentrations T by using parallel polarized incident light

$$\Gamma = \left\{2(\ln 10)n_1 \cos\theta \int_{band} OD_\parallel(\tilde{\nu})\, d\tilde{\nu}[2d_p/(2d_p - d)]^2\right\} \Big/ [3Rn_2A\{E_x^2[\sin^2\Theta(1 - \tfrac{3}{2}\sin^2\gamma_0) + \sin^2\gamma_0] + 2E_z^2[\cos^2\Theta(1 - \tfrac{3}{2}\sin^2\gamma_0) + \tfrac{1}{2}\sin^2\gamma_0]\}] \quad (5)$$

and perpendicular polarized incident light

$$\Gamma = \frac{2(\ln 10)n_1 \cos\theta \int_{band} OD_\perp(\tilde{\nu})\, d\tilde{\nu}[2d_p/(2d_p - d)]^2}{3Rn_2AE_y^2[\sin^2\Theta(1 - \tfrac{3}{2}\sin^2\gamma_0) + \sin^2\gamma_0]} \quad (6)$$

The following symbols have not been defined so far: d, thickness of sample layer; Θ, angle between molecular axis and transition moment; γ_0, angle between molecular axis and z-axis (normal to ATR plate). Equations 5 and 6 hold for liquid/micro crystalline ultrastructures. For a definition of ultrastructures cf. Fringeli and Günthard.[19]

2. Adsorption Isotherms. For a general discussion of surface phenomena the reader is referred to Adamson.[24]

2.1. Monolayer Adsorption. The adsorption of a monolayer of molecules to a surface may be described by the model of Langmuir.[25] The corresponding isotherm is

$$\Gamma = \frac{Bc}{a + c} \quad (7)$$

whereas Γ denotes the surface concentration [mol/cm²], c the concentration of the bulk solution [mol/L], B the surface concentration of a monomolecular layer, and a the ratio of the rate constants for desorption k^d [s⁻¹] and adsorption k^a [L/(mol·s)], respectively. The parameter a may be related to the enthalpy ΔH^a and entropy ΔS^a of adsorption by introducing the Arrhenius approximation

$$a = k^d/k^a = e^{-\Delta S^a/R}e^{\Delta H^a/RT} \quad (8)$$

2.2. Multilayer Adsorption. For the interpretation of the results of this work a multilayer adsorption isotherm had to be used. Van Bemmelen[26] and Freundlich[27] had used the following empirical exponential expression

$$\Gamma = \alpha c^\beta \quad (9)$$

Γ denotes the surface concentration of the adsorbed substance [mol/cm²] and c the concentration in the bulk solution [mol/L]. α and β are empirical parameters characteristic for each substance. Linearization of eq 9 results in

$$\log \Gamma = \beta \log c + \log \alpha \quad (10)$$

The approach of Brunauer, Emmett, and Teller,[28] BET-isotherm, is based on a distinct physical model, which assumes that the Langmuir eq 7 applies to each layer. However, only two different heats of adsorption are considered to be relevant, namely one (ΔH_0^a) for the adsorption of the first layer, cf. eq 8, and one ($\Delta H_1^a = \Delta H_i^a$) for all consecutive layers. The film thickness is expected to grow up to infinity as the concentration c of the solute approaches c_0. Although first developed for the adsorption of gases, the BET-isotherm (11) may be used to describe the adsorption of dissolved particles on a solid substrate, too

$$\Gamma = B\frac{bc/c_0}{(1 - c/c_0)(1 - c/c_0 + bc/c_0)} \quad (11)$$

According to eq 12, a plot of $c/(\Gamma(c_0 - c))$ vs. c/c_0 should result in a straight line

$$\frac{c}{\Gamma(c_0 - c)} = \frac{1}{Bb} + \frac{b - 1}{Bb} \cdot \frac{c}{c_0} \quad (12)$$

The parameter B denotes the surface concentration of a monolayer. It is identical with the corresponding parameter of the Langmuir isotherm (7). In order to get a better understanding of parameter b, one has to consider the two equilibrium conditions for solute adsorption to the solid substrate and to the first adsorbed layer. Equating corresponding rates of adsorption and desorption results in

$$k_0^a c S_0 = k_0^d S_1 \quad (13)$$

for the first layer and

$$k_0^d S_1 + k_1^a c S_1 = k_1^d S_2 + k_0^a c S_0 \quad (14)$$

where k_0^a, k_1^a and k_0^d, k_1^d denote the rate constants for adsorption (a) and desorption (d) with respect to the substrate (0) and first layer (1). c is the concentration of the adsorbate in solution. S_0, S_1, and S_2 are the exposed sites per unit area of free substrate surface, first layer, and second layer respectively. Denoting the total surface concentration of sites on the substrate by B it follows

$$B = \sum_{i=0}^{\infty} S_i \quad (15)$$

Equation 14 implies that the layer 1 (i) can be in a state of exchange with layer 0 ($i - 1$) and layer 2 ($i + 1$). From eq 13 it follows that the first and the last term of eq 14 may be deleted.

By introducing the Arrhenius approximation for the rate constants in eq 13 and 14 one obtains

$$A_0^a e^{-E_0^a/RT} c S_0 = A_0^d e^{-E_0^d/RT} S_1 \quad (16a)$$

(21) Fringeli, U. P. *J. Chem. Phys.* **1987**, to be published.
(22) Harrick, N. J. *Internal Reflection Spectroscopy*; Interscience Publishers: New York, London, Sidney, 1967.
(23) Fringeli, U. P. *J. Membrane Biol.* **1980**, *54*, 203.
(24) Adamson, A. W. *Physical Chemistry of Surfaces*; 4th ed.; John Wiley and Sons: New York, 1982.
(25) Langmuir, I. *J. Am. Chem. Soc.* **1918**, *40*, 1361.
(26) van Bemmelen, J. M. *Z. Anorg. Chem.* **1896**, *62*, 1.
(27) Freundlich, H. *Z. Phys. Chem.* **1907**, *57*, 385.
(28) Brunauer, S.; Emmett, P. H.; Teller, E. *J. Am. Chem. Soc.* **1938**, *60*, 309.

and

$$A_1^a e^{-E_1^a/RT} c S_1 = A_1^d e^{-E_1^d/RT} S_2 \qquad (16b)$$

A_0^a, A_0^d, A_1^a, A_1^d and E_0^a, E_0^d, E_1^a, E_1^d denote the corresponding frequency terms and activation energies. They can be related to thermodynamic expressions such as entropy of adsorption, ΔS^a, and heat of adsorption, ΔH^a, according to

$$\frac{A_0^a}{A_0^d} = e^{\Delta S_0^a/R}, \qquad \frac{A_1^a}{A_1^d} = e^{\Delta S_1^a/R} \qquad (17)$$

and

$$E_0^a - E_0^d = \Delta H_0^a, \qquad E_1^a - E_1^d = \Delta H_1^a \qquad (18)$$

Thus one obtains from eq 8, 16a, 17, and 18 for the ratio of surface concentrations of sites S_1, to sites S_0

$$y = \frac{S_1}{S_0} = \frac{c}{a_0} \qquad (19)$$

A corresponding expression is obtained for the ratio of free sites in the second and first layer. This ratio is assumed to remain constant for consecutive layers

$$x = \frac{S_2}{S_1} = \frac{S_{i+1}}{S_i} = \frac{c}{a_1} \qquad (20)$$

From eq 19 and 20 the surface concentration of free sites of the ith layer may be calculated as a function of free sites of the first layer S_1 and of the bare substrate S_0, respectively

$$S_i = S_1 x^{i-1} \qquad (i = 1, 2, \ldots) \qquad (21)$$

According to eq 15 and 21 it follows that

$$B = S_0 + S_0 y \sum_{i=1}^{\infty} x^{i-1} = S_0\left(1 + \frac{bx}{1-x}\right) \qquad (22)$$

with

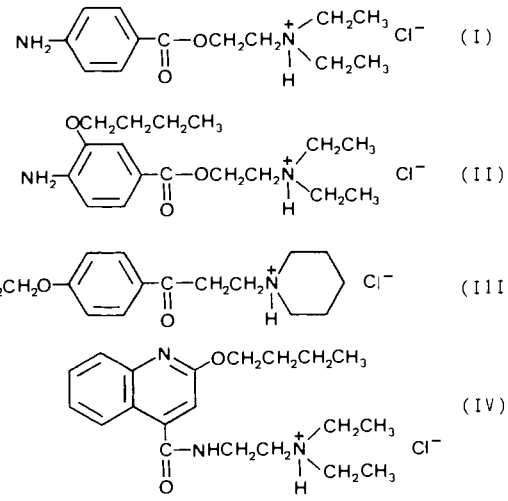
$$b = \frac{y}{x} = e^{(\Delta S_0^a - \Delta S_1^a)/R} e^{-(\Delta H_0^a - \Delta H_1^a)/RT} \simeq e^{-(\Delta H_0^a - \Delta H_1^a)/RT} \qquad (23)$$

Therefore, b depends predominantly on the difference of the heats of adsorption to the free substrate and to already adsorbed layers. Since both enthalpies are expected to be exothermic (<0), it follows that the larger the parameter b is, the more exothermic the adsorption to the substrate is compared with the adsorption to consecutive layers. Consequently, a large value of b means that sites on the bare substrate are preferably occupied as shown in Figure 4.

Materials and Experimental Procedures. IR-ATR spectra were measured on a Perkin-Elmer infrared spectrophotometer Model 580 with a Perkin-Elmer polarizer accessory (silver bromide) and an ATR attachment in the sample beam. The reference beam was equalized by a reference beam attenuator. The spectral resolution was between 2.5 and 3.5 cm^{-1} depending on the wavenumber. All spectra were performed at 25 °C. The ATR crystal was of zinc selenide (Wilks, North Haven) with a size of 52 × 20 × 1 mm. Some measurements were made with a germanium crystal of the same size. The angle of incidence was 45°, resulting in 38 active internal reflections.

1-Palmitoyl-2-palmitoleoyl-*sn*-glycero-3-phosphocholine (POPC) and dipalmitoylphosphatidic acid (DPPA) were purchased from R. Berchtold (Biochemical Laboratory, CH-3007 Berne), procaine (4-aminobenzoic acid 2-(diethylamino)ethyl ester hydrochloride) (I) and cinchocaine (2-butoxy-*N*-[2-(diethylamino)ethyl]-4-quinolinecarboxamide monohydrochloride) (IV) from Siegfried AG (CH-4800 Zofingen), oxybuprocaine HCl (4-amino-3-butoxybenzoic acid 2-(diethylamino)ethyl ester hydrochloride) (II) from Wander AG (CH-3000 Berne), and falicaine (3-(1-piperidinyl)-1-(4-propoxyphenyl)-1-propanone hydrochloride) (III) was synthesized at our institute.

The membranes were prepared with DPPA as first layer contacting the ATR plate with the polar head groups and POPC as second layer according to Fringeli.[29] DPPA was an appropriate



lipid for fixation on the plate and as a stable basis for the POPC layer. The bilayer model membrane was in direct contact with the aqueous environment consisting of a phosphate buffer in ^2H_2O adjusted to pH 7.0 and of local anesthetics dissolved at different concentrations. The buffer consisted of sodium chloride (100 mM) and phosphate (20 mM) in the form of sodium monohydrogenphosphate and potassium dihydrogenphosphate. The spectra were scanned with parallel and perpendicular polarized light 10 min after filling the solutions into the cell. It turned out that in all experiments adsorption equilibrium was reached before that time. The integrated molar absorption coefficients A of appropriate bands were determined according to eq 3 and 4. For more details the reader is referred to Fringeli.[21] In this case a Ge ATR crystal was used in order to avoid adsorption of the local anesthetics to the uncoated plate as was observed with zinc selenide ATR plates.

Results and Discussion

For determination of the behavior of the local anesthetics in the presence of the lipid model membrane the spectra were evaluated in the region between 1800 and 1400 cm^{-1}. In this region absorption bands characteristic for all four substances are expected, such as C=O stretching (ν(C=O)) from the ester or ketone and C=C stretching (ν(C=C)) from the aromatic ring, cf. formulas (I–IV).

Figure 1 shows typical polarized IR-ATR spectra of oxybuprocaine at two different concentrations (0.02 M, Figure 1b and 0.1 M, Figure 1d) interacting with a POPC model membrane in ^2H_2O buffer solution. Figure 1a presents the pure lipid membrane, whereas Figure 1 (parts c and e) are the difference spectra 1b–1a and 1d–1a, respectively.

The band at 1740 cm^{-1} (Figure 1 (parts a, b, and d)) results from the ν(C=O) of the fatty acid esters in the lipid bilayer membrane, whereas a broad background band at 1600 cm^{-1} results from the ZnSe plate. Both bands were eliminated by formation of the difference spectra Figure 1 (parts c and e). The three bands observed in the pure oxybuprocaine spectra 1c and e are assigned to ν(C=O) (1705 cm^{-1}) of the ester group and ν(C=C) (1602, 1520 cm^{-1}) of the benzene ring. It should be noted that the position of the ν(C=O) band depends on the concentration of oxybuprocaine in the bulk solution. At low concentration (0.02 M, Figure 1c) it is observed at 1710 cm^{-1}, and at high concentration (0.1 M, Figure 1e) the maximum absorbance is at 1700 cm^{-1} with a shoulder at 1710 cm^{-1}. In order to understand this phenomenon one should be aware that the membrane thickness (about 5 nm) is very small compared with the penetration depth of the ATR beam (0.9 μm at 1700 cm^{-1}). Therefore an observed absorption band is a superposition of both, the membrane adsorbed local anesthetic and the amount dissolved in the bulk solution. At low bulk concentration the membrane bound amount is dominant, whereas at high concentration the absorption from the bulk

(29) Fringeli, U. P. *Chem. Phys. Lipids* **1987**, to be published.

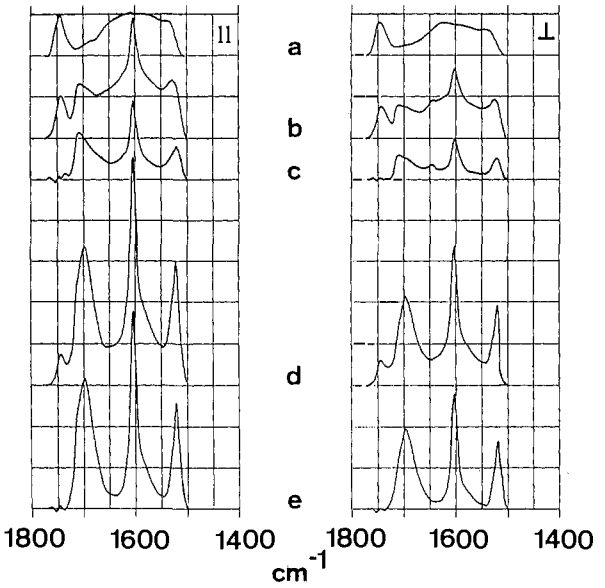


Figure 1. IR-ATR spectra of oxybuprocaine adsorbed to a POPC model membrane (parallel (||) and perpendicular (⊥) polarized incident light, absorption spectra, $\theta = 45°$, ZnSe plate, $T = 25$ °C, 20 mM phosphate buffer, pH 7.0, with 100 mM NaCl): (a) spectra of the POPC model membrane; (b) spectra of the POPC membrane in a solution of 0.02 M oxybuprocaine; (c) difference spectra b–a, absorption of oxybuprocaine; (d) spectra of the POPC membrane in a solution of 0.1 M oxybuprocaine; (e) difference spectra d–a, absorption of oxybuprocaine.

solution dominates the corresponding band. The band shift of about 10 cm^{-1} observed upon adsorption of oxybuprocaine to the POPC membrane reveals that the conformation and/or the microenvironment of the ester group is different in the adsorbed and dissolved state, respectively. A corresponding response of benzene ring stretchings was too small to be detected with a spectral resolution of about 3 cm^{-1}.

In order to separate the superimposed spectra of adsorbed and dissolved local anesthetic, one has to measure the spectrum of the corresponding solution in absence of the lipid membrane. For spectral subtraction (cf. Figure 1), however, one has to consider that the magnitude of the electric field component normal to the plate (E_z) in the rarer medium depends on whether the bulk solution is in direct contact with the ATR plate (refractive index n_1) or with a thin layer coating at the ATR plate (membrane, refractive index n_2). According to Fringeli and Günthard[19] a direct subtraction is only possible with spectra obtained with perpendicular polarized light (cf. eq 25). In order to evaluate the parallel polarized spectrum of membrane bound local anesthetics the spectrum measured from the bulk solution (refractive index n_3) in the absence of a POPC membrane has to be multiplied by a factor according to eq 24 before subtraction from the overall spectrum can be performed

$$\Omega_{\parallel}^{iso}(\text{thinlayer}) = \Omega_{\parallel}^{iso}(\text{bulk}) \frac{E_x^2 + E_z^2(\text{thinlayer})}{E_x^2 + E_z^2(\text{bulk})} \quad (24)$$

$$\Omega_{\perp}^{iso}(\text{thinlayer}) = \Omega_{\perp}^{iso}(\text{bulk}) \quad (25)$$

where $\Omega_{\parallel,\perp}^{iso}(\text{bulk})$ denotes the integrated optical densities ($\Omega = \int OD(\tilde{\nu})\, d\tilde{\nu} = -\int \log T(\tilde{\nu})\, d\tilde{\nu}$) of a band resulting from an isotropic bulk solution in contact with the ATR plate for parallel (||) and perpendicular (⊥) polarized incident light. $\Omega_{\parallel,\perp}^{iso}$ (thin layer) is the integrated optical density of the same solution in contact with a thin either non- or low-absorbing layer coating the ATR plate. Figure 2 shows the plots of integrated optical densities of oxybuprocaine at several concentrations. The solid lines denote the total amount of the anesthetic as revealed by the original spectra, i.e., the superposition of membrane bound and dissolved local anesthetic. Dotted lines represent the linear fit according to Lambert–Beer's law of experiments in absence of the model membrane. Correlation coefficients were 0.997 for parallel and

Table I. Integrated Molecular Absorption Coefficients A

substance	vibration	wavenumber, [cm^{-1}]	A [cm/mol]
cinchocaine	ν(C=O)	1645	9.658×10^6
	ν(C=C)	1600	7.621×10^6
falicaine	ν(C=O)	1664	1.061×10^7
	ν(C=C)	1600	1.057×10^7
oxybuprocaine	ν(C=O)	1700	1.923×10^7
	ν(C=C)	1600	1.289×10^7
procaine	ν(C=O)	1690	2.134×10^7
	ν(C=C)	1605	2.113×10^7

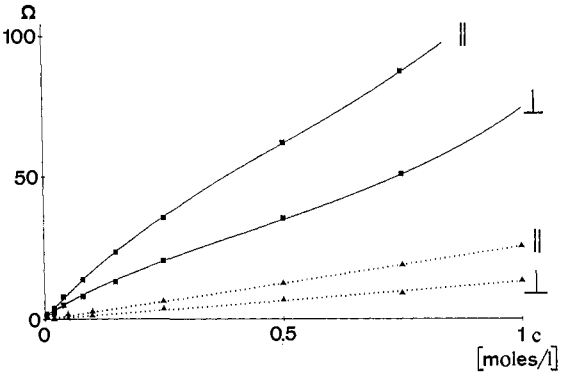


Figure 2. Integrated optical densities of oxybuprocaine solutions Ω as a function of the bulk concentration c (value of Ω cf. eq 24 and 25): solid lines, in contact with a POPC model membrane; dotted lines, solutions in absence of the membrane.

0.996 for perpendicular polarized light and demonstrate that Lambert–Beer's law is applicable in our concentration range. Corresponding results were obtained with procaine, falicaine, and cinchocaine.

The slopes $k_{\parallel}$ and $k_{\perp}$ of the dotted lines were used to calculate the integrated molar absorption coefficients A of ν(C=O) and ν(C=C) which is a prerequisite for the determination of the surface concentration of the local anesthetic on the membrane (cf. eq 5 and 6). The results, an average of the measurements with parallel and perpendicular polarized light, respectively, are shown in Table I.

The difference of optical densities of the total amount of anesthetic minus the bulk solution (taking eq 24 and 25 into account) reveals the amount of local anesthetic adsorbed to the lipid membrane. The corresponding surface concentrations were calculated as a function of the bulk concentration by means of eq 5 and 6. It was assumed that the integrated molar absorption coefficients A of the local anesthetics were the same for the adsorbed and the dissolved state, respectively.

The adsorption isotherms as presented in Figure 3 show unambiguously that all four local anesthetics under consideration tend to adsorb in multilayers on the lipid membrane even at pharmacologically relevant concentrations. e.g., procaine, the local anesthetic with weakest activity is usually applied as solutions containing 2–3% by weight, i.e., 0.07–0.11 M. As concluded from Figure 3, a bulk concentration $c = 0.1$ M corresponds to a mean surface concentration of $\Gamma = 2.9 \times 10^{-9}$ mol/cm^2 of procaine. On the other hand, molecular model considerations result in a monolayer surface concentration of $\Gamma_m = 1.6 \times 10^{-10}$–$3.3 \times 10^{-10}$ mol/cm^2, depending on the conformation of procaine in the layer. It follows that at a bulk concentration of 0.1 M the mean of layers covering the membrane ranges from 9 to 18. Multilayer formation is observed with the other local anesthetics investigated in this work, although they are applied pharmacologically at lower concentrations (oxybuprocaine, 1.15×10^{-2} M; falicaine, 8.0×10^{-3} M; cinchocaine, 2.6×10^{-3} M).

Evidently, there is a correlation between multilayer thickness at a given concentration and local anesthetic activity. For comparison the pharmacological data of the local anesthetics used in this work are shown in Table V. Since there is no standardized testing method for local anesthetics, a comparison of activities

Table II. Parameters of the BET Isotherms[a]

substance	B [mol/cm^2]	b	c_0 [mol/L]
cinchocaine	9×10^{-9}	30	0.9
falicaine	$1.17 \times 10^{-8} \pm 2.4 \times 10^{-10}$	$11.02 \pm 5.9 \times 10^{-4}$	$1.26 \pm 3.9 \times 10^{-2}$
oxybuprocaine	$1.19 \times 10^{-8} \pm 1.2 \times 10^{-10}$	$11.18 \pm 4.6 \times 10^{-4}$	$1.73 \pm 8.4 \times 10^{-3}$
procaine	$1.50 \times 10^{-8} \pm 4.3 \times 10^{-11}$	$7.00 \pm 9.4 \times 10^{-4}$	$3.09 \pm 2.1 \times 10^{-3}$

[a] Cf. eq 11.

determined by different workers is very difficult. Therefore, the data shown in Table V are the average of several values found in literature.[30-32]

A more detailed insight in local anesthetic action is obtained via a least-squares analysis with respect to the parameters B, b, and c_0 of the BET eq 11. The results are presented in Table II and plotted (solid lines) in Figure 3. The dotted line denotes a graphic extrapolation for expected surface concentrations of cinchocaine for a hypothetic solubility over the whole concentration range. At about 0.01 mol/L, the saturation point of cinchocaine is already reached, as observed by a precipitation and a discontinuity in the Γ vs. c curve. Further enhancement of cinchocaine concentration leads to a redissolution by micelle formation above approximately 0.1 mol/L. This process is paralleled by a sudden detachment of the membrane from the ATR plate. This phenomenon is also observed with the other local anesthetics, however, only at very high concentrations (>1 M). Therefore a determination of the BET parameters by least-squares analysis was not possible for cinchocaine. Nevertheless, the parameters B and b which are responsible for the shape of the curve at low concentrations (i.e., where reasonable experimental data of cinchocaine are available) could be determined empirically with good reliability while c_0 was found to be within 0.8 and 1.0 mol/L. In any case this value is smaller than the corresponding values of falicaine, oxybuprocaine, and procaine. The mean of 0.9 mol/L was chosen for presentation in Figure 3a. For falicaine, oxybuprocaine, and procaine the agreement between the experimental values and the BET adsorption isotherm is good (cf. Table II). Linearized presentations of experimental data according to the Freundlich isotherm, eq 10, and to the BET isotherm, eq 12, are shown in Figure 3 (parts b and c), respectively. Although the fit to the two parameter expression of Freundlich correlates well (cf. Table III), we preferred the three parameter BET expression because on the one hand Figure 3b reveals a systematic deviation of the data from theory, and, on the other hand, all BET parameters have a well defined physical meaning, in contrast to the parameters of the Freundlich isotherm. The results of the linear regression calculations are collected in Tables III and IV. Moreover, it should be mentioned that BET parameters can be reasonably correlated to pharmacological activities, which is not evident for the Freundlich parameters.

The BET parameters b and c_0 (cf. eq 11) turned out to be most relevant. According to eq 19, 20, and 23, b is a measure for the affinity of the solute to adsorb to the free substrate (membrane) and to already adsorbed layers, respectively. e.g., $b = 1$ means that the corresponding heats of adsorption are approximately equal, whereas for $b > 1$ the heat of adsorption to the membrane is expected to be more exothermic than that to condensed layers. Therefore, a large value of b means that free sites on the membrane surface are more attractive for the solute than free sites on the adsorbate. Consequently, a local anesthetic with a large b value tends to complete the first monolayer (i.e., to seal the membrane) at lower bulk concentration than a local anesthetic with a lower b value. In order to visualize this fact, we have calculated possible surface coverage profiles for the compounds under consideration (Figure 4). This hypothetic example is based on the assumption that 90% of the membrane surface shall be covered by the local anesthetic, i.e., $S_0/B = 0.1$ according to eq 22. Since b is known for each compound (cf. Table II), the parameters x and y can be calculated by means of eq 22 and 23. The surface profiles as shown in Figure 4 have been calculated by means of eq 19 and 21. They demonstrate that a potent local anesthetic should tend to complete the first monolayer on the membrane rather than to form multilayered aggregates on the membrane. In the framework of the BET theory this property

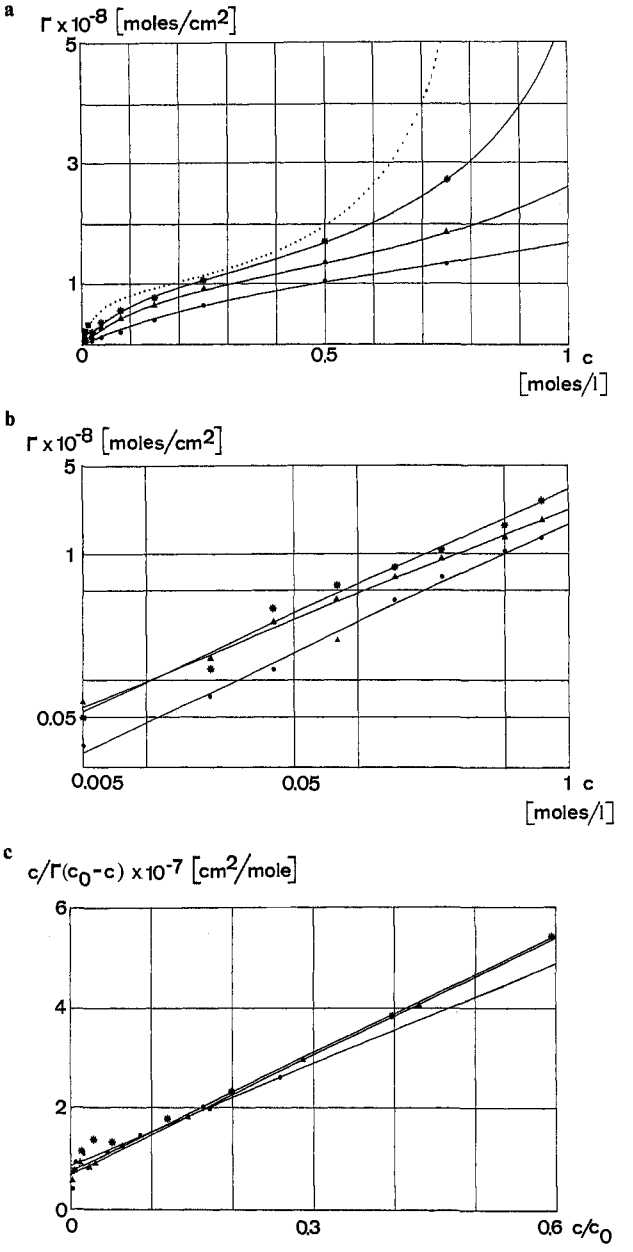


Figure 3. Adsorption isotherms of procaine (●), oxybuprocaine (▲), falicaine (*) and cinchocaine (■) (20 mM phosphate buffer, pH 7.0, with 100 mM NaCl). Surface concentrations Γ have been calculated with eq 5 and 6: (a) Linear plot of surface concentration Γ vs. bulk concentration c. The solid lines are obtained via a least-squares fit with respect to the BET eq 11. The dotted line is the empirical extrapolation ot the BET isotherm of cinchocaine (membrane detachment at $c > 0.01$ M); (b) linearization of the isotherm according to Freundlich (eq 10); (c) linearization according to BET (eq 12). c_0 is the critical concentration for condensation in the BET model.

(30) Büchi, J.; Stünzi, E.; Flury, M.; Hirt, R.; Labhart, P.; Ragaz, L. *Helv. Chim. Acta* **1951**, *34*, 1002.
(31) Profft, E. *Chem. Tech.* **1952**, *4*, 241.
(32) Schiltknecht, J. Ph.D. Thesis ETH Zürich, 1987, in preparation.

Table III. Parameters of the Isotherms According to Freundlich[a]

substance	α	β	correlation
falicaine	3.33×10^{-8}	0.770	0.994
oxybuprocaine	2.30×10^{-8}	0.686	0.996
procaine	1.74×10^{-8}	0.792	0.997

[a] Cf. eq 9 and 10.

Table IV. Linearized BET Isotherms[a]

substance	c/c_0	$c/\Gamma(c_0 - c)$ [cm²/mol]	correlation
falicaine	7.84×10^7	7.46×10^6	0.999
oxybuprocaine	7.86×10^7	6.89×10^6	0.994
procaine	6.70×10^7	8.68×10^6	0.974

[a] Cf. eq 12.

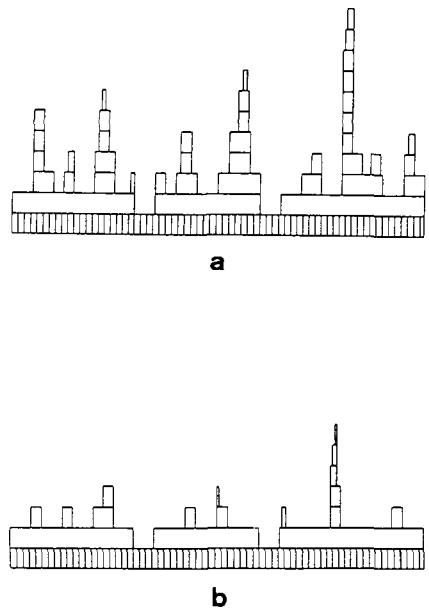


Figure 4. Adsorption of local anesthetics to the model membrane. Calculated surface coverage profiles according to the BET model under the assumption of a 90% membrane coverage by the local anesthetic: (a) procaine (b = 7.00, c_0 = 3.09, weak activity); (b) cinchocaine (b = 30, c_0 = 0.9, high activity). In order to achieve the same mebrane sealing effect the concentration of the high active local anesthetic may be 8.2 times lower than that of the low active (cf. eq 26).

is expressed by the parameter b. Furthermore, it follows from eq 22 that completion of the first layer ($S_0 = 0$) requires $x = 1$. As concluded from eq 19 and 20 $x = 1$ also means that consecutive layers are completed, i.e., that the condensation concentration c_0 is reached. Thus it follows that x is related to the bulk concentration c by

$$x = S_{i+1}/S_i = c/c_0 \qquad (26)$$

Therefore, one may conclude that the membrane sealing property of a local anesthetic is enhanced by the larger b and the smaller c_0.

Finally it should be mentioned, that no distinct interpretation of the parameter B can be given so far. Although B has a simple meaning as monolayer surface concentration in the BET theory (eq 11 and 22) the values determined by least-squares analysis of our data (cf. Table II) are found to be more than one order of magnitude too large. The relative magnitudes, however, are consistent with the molecular dimensions of the compounds. As expected, B is of minor importance for the characterization of local anesthetic action. Nevertheless, one might try to obtain more realistic B values by expanding the BET model distinguishing three different regions of interaction instead of only two. The first one is adsorption to the bare membrane, the second one is adsorption to an inner region of tightly packed local anesthetic, and the third one is adsorption to a loosely packed outer region of adsorbed local anesthetic. This idea is also supported by the systematic deviation of experimental data in the linearized BET plot (eq 12, Figure 3b) at low concentrations and by a recent NMR study by Kelusky et al.[11]

Table V. Pharmacological Activities

substance	relative surface anesthetic activity	relative conductive anesthetic activity
cinchocaine	2000[30]	15[30]
falicaine	200[31]	11[32]
oxybuprocaine	400[30]	10[30]
procaine	1[30]	1[30]

Conclusions

The four local anesthetics procaine, oxybuprocaine, falicaine, and cinchocaine were shown to adsorb in multilayers to a lipid bilayer membrane (POPC) already at pharmacologically relevant bulk concentrations. This fact strongly points to an unspecific mechanism of local anesthesia by sealing the membrane as was suggested first by Büchi.[15] More recently local anesthetic adsorption to membranes was also reported by Ohki.[33] Moreover it was found from least-squares analysis of the data with respect to the BET isotherm (eq 11) that the two parameters b and c_0 can be used to characterize the efficiency of a local anesthetic (cf. Table II). Thus, it is concluded that a high performance local anesthetic should exhibit BET parameters $b > 10$ and $c_0 < 1$ mol/L. As visualized by Figure 4, such a compound has a distinct tendency to adsorb in flat compact areas rather than in thick multilayered islands. The former is a prerequisite for efficient membrane sealing at low bulk concentration.

It is not necessary to discard mechanisms proposed earlier: interaction with the lipids and a following change of the physical properties of the membrane[1,14] or direct interaction with proteins and change of their conformation.[13] However, we consider membrane sealing described above as the principal mechanism of local anesthetic action. In addition, based on our results a specific interaction with receptors can be excluded.

Pharmacological relevance of these results is given since all spectroscopic measurements have been performed in aqueous environment and in a bulk concentration range of 1 mM (which is significantly below the pharmacological concentrations (cf. Table V) up to 0.75 M. Cinchocaine, the most active local anesthetic investigated in this work, is known to be toxic at concentrations above 5×10^{-2} M. Experimentally, this compound was found to form micelles at concentrations above $\simeq$0.1 M. As mentioned in the section above, the lipid bilayer membrane detaches from the ATR plate at the moment of micelle formation. Thus, dissolution of the membrane might be an indication of the toxicity of cinchocaine at higher concentrations.

The results presented in this paper demonstrate the applicability of infrared ATR spectroscopy for relevant in situ studies of drug–membrane interaction. Such studies are not at all limited to unspecific interactions as in the case of local anesthetics. It can also be used for molecular studies of the interaction of drugs or other ligands with immobilized receptors or enzymes.[34] Finally, it should be mentioned that experiments as performed in this work can give substantial support in the development of new drugs and therefore should help to reduce experiments with animals at least in the prescreening state.

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Registry No. I, 59-46-1; II, 99-43-4; III, 1155-49-3; IV, 85-79-0.

(33) Ohki, S. *Biochim. Biophys. Acta* **1984**, *777*, 56.
(34) Fringeli, U. P.; Ahlström, P.; Vincenz, C.; Fringeli, M. *SPIE* **1985**, *553*, 234.