Review

Interactions of some local anesthetics and alcohols with membranes

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Abstract

A review of the results obtained by our group in the last decade regarding the interactions of procaine, lidocaine, dibucaine and tetracaine with membranes is presented in the context of the literature data. The action upon membranes, in first approximation monomolecular film of stearic acid spread at the air/water interface used as a membrane model, the modification of biomembrane structure and function using diffraction methods, lipid phase transition, fluidity of lipids and proteins, membrane expansion and platelet aggregation were studied. The thermodynamic knowledge of membrane-alcohol interactions improved by using highly sensitive calorimetric techniques are briefly reported. One of the main conclusions is that the physical state of a monolayer model membrane was the result of competitive interactions between film-film and film-substrate interactions. It was taken into account that local anesthetics, such as lidocaine, carbisocaine, mesocaine, showed changes in the bilayer structure, reflected in macroscopic mechanical properties. This restructuring of the lipid bilayer has a significant influence on the operation of functional subunits, e.g. ionic channels formed by gramicidin. The results support the concept of non-specific interactions of local anesthetics with lipid bilayers. The theoretical modeling of the interactions of local anesthetics is closely compared with experimental data. Our new theory of relaxation for these interactions is using a non-archimedean formalism based on a process resulting from superpositions of different component processes which take place at different scales of time. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Anesthesia was discovered more than a century ago but the mechanism of action of anesthetics is still unknown and subject of intense experimental and theoretical work. Although it is now well established that anesthesia is brought about by a large number of chemically different molecular species, their exact sites of action and details of their interactions with their molecular components of nerve cells are still subject of considerable controversy. Consequently, especially during the last two decades, a significant amount of interdisciplinary scientific research has been devoted worldwide to the task of obtaining a unified picture of anesthesia mechanism. Attempts to solving this problem can be ascribed to a variety of disciplines, among which chemistry, biophysics, biochemistry, physiology and pharmacology are the most important. These approaches have produced a remarkable amount of scientific data, including both physico-chemical and pharmacological characteristics of various compounds with anesthetic action as well as information on their interactions with different biological and model systems. The investigation itself and the collection and interpretation of information goes beyond the capacity of any individual investigator or labora-
tory. Therefore, naturally, appeared the excellent idea of Kenji Ogli to publish a new international journal “Progress in Anesthetic Mechanism” [1] in 1993 and to organize, periodically the International Workshop on Anesthetic Mechanisms, the second edition, held in 1999. The results achieved thus far have been extensively reviewed [2–11]. Numerous models and theories have been put forward for anesthesia, which are based mostly on biophysical and biochemical data [12–18]. The theories of molecular mechanism of anesthesia: lipid theories, perturbation of physicochemical properties of biomembranes, theories involving the interaction of anesthetics with water, protein theory [19] along with the chemicals that could exert their modulation effect through the lipid domain on transmembrane protein structure and function [20] are particularly well presented.

The terms “local anesthesia” or “regional anesthesia” are used to designate the loss of sensation in a more or less discrete area of the body. Unlike general anesthesia, this loss of sensation is not accompanied by any simultaneous loss of consciousness [21]. All chemical compounds, which are used as local anesthetics, are capable of blocking the initiation or conduction of nerve impulses. However, it should be noted that not all the substances displaying such properties have been accepted for clinical use as local anesthetics [22]. Historically, the first chemical used as local anesthetic was cocaine. Since this discovery of local anesthesia in 1884 by Sigmund Freud in Heidelberg, the search for the replacement by safer and more reliable anesthetic agents became stringent due to the strong addicting liabilities of cocaine. The first man-made local anesthetic agent by chemical synthesis, in 1905, was procaine (P). The following local anesthetic to be synthesized in 1943 was lidocaine. Its versatility and combination of favorable properties soon made it most widely used local anesthetic [21,22]. Many chemicals have been proposed as local anesthetics [23,24] and search for even better local anesthetics has continued up to date. As an example, it is worth mentioning the synthesis of 4-alkylpiperazinoeth esters of o-heptyloxyphenylcarbamic acid, which are two orders of magnitude more active than P or lidocaine [25].

Most of the local anesthetics now in use are amphiphilic molecules, i.e. molecules containing both, hydrophilic group (usually consisting of an amino moiety) and a hydrophobic (or lipophilic) group (generally containing an aromatic ring). The hydrophobic groups are involved in the diffusion and binding of local anesthetic molecules to cell membranes, while the hydrophilic ends confer to these molecules their water solubility characteristics and their ability to spread into the tissues. The presence of an electron donor group in the molecule seems to increase both biological activity and toxicity. The two end groups are usually connected by an alcohol chain with 2–5 carbon atoms in length. The distance of 6–9 Å is critical. Longer molecules are characterized by improved biological activities and liposolubilities [26].

Among the compounds displaying such properties, the tertiary amine type molecules deserve a special mention, e.g. P, lidocaine, dibucaine, tetra- caine, butacaine, etidocaine, proparacaine, propoxycaine, etc. The main reason for this special interest from the scientific community could be attributed on one hand to the attempts at elucidating the exact molecular mechanism by means of which they exert their anesthetic action. On the other hand, substantial scientific evidence has been accumulating, which shows that, when interacting with living systems, biomolecules aggregates, or even isolated biomolecules, tertiary amines produce effects which can be regarded either as secondary effects, or as effects having nothing in common with their ability to produce local anesthesia. These new effects range from changes induced in bacterial gene expression to modifications of biomembranes molecular structure and permeability properties, changes of enzyme activities, immune response, ultrastructure of subcellular fraction, etc. Among these effects, an explicit mention should be made of controversy regarding the favorable consequences of the systemic long-term treatment of the aging process and the chronic diseases of the elderly with low doses of P based drugs [27–35].

The mechanism of action of the local anesthetics upon excitable membranes, which appears to be well established so far is (1) diffusion of the uncharged form of the local anesthetic across the
nerve sheath and nerve membrane; (2) re-equilibration between the uncharged and cationic forms on the axoplasmic surface of the nerve membrane; and (3) penetration into and attachment to a receptor at a site within the sodium channel [24,36,37]. Debate has focussed on whether such sites are purely lipid in nature or whether protein targets may be involved [38–40].

Unsuccessful results of the research done until today regarding the mechanism of action of anesthetics may be related and to the elusive nature of the site of interaction of so many compounds that differ in their chemical structure but share among each other their hydrophobic character. Thus, the subject covers a broad range of chemical compounds such as aliphatic hydrocarbons, alcohols, ethers and halogenated compounds as well as their interaction phenomena.

Our research was mainly focussed on local anesthetics e.g. P, lidocaine, dibucaine, tetracaine, and the results refer almost exclusively to these drugs. The aim of this review is therefore limited to making a general presentation of the results obtained by our group in this field in the last decade, in the general context of the literature data.

2. Local anesthetics and interfacial phenomena

According to some models, anesthesia can be regarded as an interfacial phenomenon involving molecular processes that occur at the interface between biomembranes and their aqueous environment. One possible cause of anesthesia is now considered to be the relative increase in the hydrophobicity of the interface between surrounding water and membranes, as a result of the partitioning of anesthetic molecules between these microenvironments with different polarities [15]. The monolayer/substrate interactions, in the studies of the possible mechanisms of anesthesia, have provided and will continue to provide much useful information. Since the physical state of any monolayer must result from competitive interactive contributions between the membrane components, and between the membrane components and the substrate, it is important that the substrate additive to be studied both, in the presence and the absence of the monolayer.

A recent review on surface and colloidal properties of some local anesthetics P, tetracaine, dibucaine, bupivicaine, mepivicaine and lidocaine presents their molecular structure and dissociation equilibrium, the surface adsorption, volume behavior in water, Krafft phenomenon on the coagel–micelle transition aggregation characteristics, surface activity and local anesthetic potency [11].

We reported the action of P upon membranes, in first approximation the monomolecular film of stearic acid (SA), spread at the air/water interface which was used as a membrane model [41–54].

Model membrane studies using monomolecular films [55,56], named also Langmuir monolayers, at the air/water interface have demonstrated the adsorption and penetration of anesthetics into the lipid membrane producing an increase of compressibility which is dependent on both, the lipid composition of the membrane and the precise nature of the drug. The experiments with Langmuir monolayers have an advantage over some other model systems since the molecular arrangements and orientation can be principally well controlled by changing the monomolecular area and surface pressure of the biosurfactant monolayer [57,58].

Our research has shown that P interacts specifically with a monomolecular Langmuir membrane formed by fatty acids SA and L-α-distearoylphosphatidylcholine (DSPC) [41,42,46,51,59]. SA was chosen for several reasons. Firstly, its monolayers are stable in a large range of pH [58]. Secondly, the SA monolayers are very sensitive to the subphase electrolytes [41,58].

The compression isotherms, i.e. surface pressure (π) vs. mean molecular area (A) curves have been recorded by using the Wilhelmy method. SA had been spread on different aqueous subphases: 10−2 M HCl for pH = 2, twice distilled water and phosphate buffer solution with pH = 8, respectively. Compression isotherms have been recorded both in the absence of P and by dissolving P in the aqueous subphase ensuring a P concentration of 10−3 M.
Since both film materials, SA and P, may participate in protolitie equilibria, diagrams have been constructed giving the fraction $x$ of the different molecular species as function of pH (Fig. 1).

In the case of SA, calculations have been performed by using the surface acidity constant $pK_a = 5.63$ [59]. As it can be seen from Fig. 1(a), at pH = 2, SA is completely unionized and forms an uncharged film. In the case of unbuffered subphases, the pH of twice distilled water is 5.6, while that of $10^{-3}$ M P solution is 5.2. Consequently, beside the neutral SA molecules, the monolayer contains amount of stearate anions. At pH = 8, SA is completely ionized and gives a charged film containing only stearate anions.

Procaine is a tertiary amine containing also a primary amine group linked to an aromatic ring. Therefore, it may exist as a neutral molecule (P), monocation ($PH^+$) and dication ($PH^{2+}$). The $pK_a$ of the monocation has been found [48] to be $pK_a = 8.865$ and that of the dication $pK_a = 1.95$. Our calculations revealed (Fig. 1(b)) that at pH = 2, the ratio of $PH^{2+}$ amounts to about 47% with a rest of 53% for the $PH^+$. In the case of unbuffered subphases the only one species present is $PH^+$. At pH = 8, $PH^+$ is the predominant species, but there are also about 12% of neutral molecules.

Fig. 1. Fraction of molecular species as function of pH. (a) SA; (b) P.

The effect of subphase P upon the compression isotherm (Fig. 2) has an expanding influence on the monolayer, i.e. at a given $\pi$ value $A$ is higher in the presence of P, indicating a penetration of P molecules into the SA monolayer.

In the same time an important increase of the collapse pressure is observed, i.e. in the presence of P the monolayer becomes more stable.

Fig. 2. Compression isotherms of SA monolayers: curves (1) and (2) – unbuffered subphase of pH = 5.6 and pH = 5.2, respectively; curves (3) and (4) – pH = 8; (1) and (3) – (P) = O; (2) and (4) – (P) = $10^{-3}$ M.

Fig. 3. Molecular area increment ($\Delta A$) due to the subphase P; curve (1) pH = 2; (2) pH = 5.2; (3) pH = 8.
By constructing the area increment ($\Delta A$) vs. $\pi$ curves (Fig. 3) it may be observed that at compression $\Delta A$ the penetration of P increases. At high $\pi$ values, $\Delta A$ vanishes, i.e. the penetrated P is squeezed out from the monolayer, probably by forming a monolayer of P cations, leading to the stabilization of the SA monolayer and to its enhanced collapse pressure.

One may see further that the expanding effect of P increases with a higher pH and becomes very important in alkaline media.

The $P$ penetration into SA monolayer [46,52] is characterized by the penetration number $n_p$ defined as the number of drug molecules $n_2$ divided by the number $n$ of SA molecules.

$$n_p = n_2/n_1.$$ (1)

This penetration number may be correlated to the area increment $\Delta A$, viz.

$$n_p = \Delta A/(A_{10} - A_1 + A_2).$$ (2)

where $A_{10}$ stands for the mean molecular area in the absence of P, i.e.

$$A_{10} = (n_0A_0 + n_1A_1)/n_1,$$ (3)

where $n_0$ and $n_1$ stand for the number of water and SA molecules, respectively, in the monolayer, $A_0$ and $A_1$ are the area necessity of a water and SA molecule, respectively and $A_2$ means the area necessity of the P molecule.

Penetration number value has been derived from the compression isotherms, by using Gibb’s equation [60]. The adsorption of the subphase component 2 at the air/water interface in the presence of an insoluble monolayer forming component 3 obey the following relation:

$$\Gamma_2 = \frac{1}{kT} \left( \frac{\partial \pi}{\partial \ln c_2} \right)_{T,A_3},$$ (4)

at constant $T$ for constant mean molecular area $A_3$ of the insoluble surfactant. The penetration number $n_p$ is equal to:

$$n_p = \frac{A_3 - \bar{A}_3}{kT} \left( \frac{\partial \pi}{\partial \ln c_2} \right)_{T,A_3},$$ (5)

where $A_3$ stands for the partial molecular area of the component 3. By taking for $A_3$ the collapse area of SA, $n_p$ values were calculated as function of $A_3$.

### Table 1

<table>
<thead>
<tr>
<th>Subphase</th>
<th>Maximum $n_p$ value; $c_2$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^{-3}$</td>
</tr>
<tr>
<td>pH 2</td>
<td>0.040</td>
</tr>
<tr>
<td>Unbuffered</td>
<td>0.059</td>
</tr>
<tr>
<td>pH 8</td>
<td>0.113</td>
</tr>
</tbody>
</table>

The $n_p$ vs. $A_3$ curves allowed us to have an insight into the conformational changes of P during the compression of the SA monolayer. Eq. (2) describes very well the experimental $n_p$ vs. $A_3$ curves if for the variation of $A_3$ one presumes that at the spreading of the SA monolayer, the P molecules are adsorbed in the interface in a horizontal lying down position. At compression of the monolayer, they gradually adopt a vertical position and this process is completely achieved at about $\pi = 26$ mN/m, corresponding to the liquid condensed to solid order phase transition of the SA monolayer.

Penetration number values increase with increasing pH, especially in the alkaline region as seen from the maximum $n_p$ values given for $10^{-3}$ and $10^{-2}$ M of P solutions, illustrated in Table 1.

This is due to the protolitic equilibria in which participate both P and SA. Increasing pH entails gradual deprotonation of $PH_2^+$ into $PH^+$ and P, and consequently increases the surface activity of the soluble surfactant. On the other hand, the deprotonation of the SA molecules makes easier the penetration of the P cations into negatively charged SA monolayers.

We performed similar studies by using instead of SA, DPPC, DSPC, MA and cholesterol (C), also mixed monolayers containing C and DPPC as insoluble films forming the surfactant. It is interesting to emphasize that at the same concentration, $n_p$ increases in the order $C < SA < DSPC < DPPC$.

Similar investigations of the behavior of other local anesthetics (e.g. dibucaine, tetracaine) at lipid/water interfaces were also carried out by other laboratories [61–64].
Another type of simple experimental models investigated by us consisted of liquid membranes at the interfaces between immiscible liquids and an aqueous solution containing a surface active agent, such as nitrobenzene. In the presence of adequate solutes, these relatively simple systems develop spontaneous pH and/or electrical potential oscillations, which are associated with the hydrodynamic instabilities occurring at the interface. These simple systems show selective permeability properties apart from their ability to develop potential oscillations, which makes them, up to a certain point, analogs of biological membranes. Our experiments revealed that in the functioning of these liquid membranes, the development of potential oscillations were stimulated by the presence of P, dibucaine and tetracaine [65–68]. Other investigations have been done on the effects of P, tetracaine and lidocaine on self-sustained electrical oscillations for a lipid membrane comprising dioleyl phosphate. This membrane exhibits oscillation of the membrane potential in a manner similar to that of nerve membrane [69,70].

A satisfactory explanation of the molecular mechanisms of interaction between local anesthetics and liquid membranes requires further investigations.

3. Effects of alcohols on membranes

The fundamental thermodynamic information about the properties of anesthetic molecules is incomplete regarding the molecular mechanism of local anesthesia [71].

Alcohols that form hydrogen bonds with water molecules [72] are generally thought to disrupt normal membrane function by penetrating into membrane domains and hydrophobically interacting with the membranes [73–75].

Despite the controversy on membrane fluidizing theory of anesthesia [76,77], or the questioning problem if alcohols really fluidize the erythrocyte membrane [78], an enhance of the membrane fluidity with the alcoholic concentration was observed [79].

3.1. Model membrane/substrate interactions: ethanol and procaine interactions

The property of ethanol to lower the surface tension of water plays a major role in its ability to affect membrane lipids [80]. Typical lipids show little expansion on exposure to ethanol substrate and may even show condensation. At the same time, the overall stability of the lipid phase is significantly reduced.

Our general conclusion (see chap. II), [50–54], and also of other laboratories [80–82] was that the physical state of a monolayer model membrane was the result of competitive interactions between film–film and film–substrate interactions.

The role of ethanol is one of considerable interest and throws particular light on substrate interactions. It was found [83] that SA films underwent condensation on ethanol substrates and also that the liquid condensed/solid condensed transition and the collapse pressure moved to lower surface pressure (Fig. 4).

**Fig. 4.** The surface pressure (\(\Pi\)) vs. area/molecule (4\(A\)) isotherm of SA at \(pH = 2\). The horizontal broken lines at 2.18 and 26 mN/m indicate the surface tension reduction for aqueous ethanol substrate of 0.1, 1.0 and 2.0 M ethanol concentrations. The condensations observed [83] may be reproduced by resetting the zero of the ordinate at the various ethanol substrate surface tension reductions.
Measurement of the surface tensions of the appropriate ethanol substrate [83] confirmed that this alone could produce the reported condensation. In other words, ethanol may both penetrate film molecules and lower the substrate surface tension. Depending on which effect predominates, film molecules may exhibit either expansion or condensation. Procaine exhibits surface and line activity at physiologically significant subphase concentrations and is capable of penetrating lipid films in cationic and neutral forms with the neutral forms being most effective.

3.2. Thermochemistry of alcohol–membrane interactions

Thermodynamic knowledge of membrane–alcohol interactions has recently been improved through the use of modern, highly sensitive calorimetric techniques [84–91]. The general picture of this work suggests that the transfer from water to phospholipid bilayer carries a positive enthalpy change (i.e. is endothermic) for small (C2–C6) monohydric alcohols, while it become negative (exothermic) for large (C7–C9) monohydric alcohols [85–87]. For the small alcohols it was shown that the enthalpy of water–membrane transfer was similar to that observed for water–oil partitioning, and this suggests that the energetics of alcohols binding to a phospholipid membrane is dominated by the dehydration of the alcohol molecule, rather than direct contributions from specific alcohol–membrane contacts [86–91].

The thermodynamics of alcohol–membrane interactions are highly dependent on temperature. In particular, a strong positive anomaly in the heat of interaction was observed around the main transition temperature of the lipid bilayer. Parallel measurements of this interaction enthalpy and the partitioning coefficient revealed that this anomaly arose almost entirely from addition adsorption of alcohol into the membrane, while the enthalpy of the membrane associated alcohol molecules showed little temperature variation. A likely origin for the anomaly is the formation of fluctuating gel and fluid domains in bilayer membranes close to main transition temperature [89,90,92]. Meticulous analysis of the temperature and composition dependence also showed that the hydrophobic effect was the major driving force for membrane–alcohol association [85,89]. Based on these observations, the partitioning of alcohol into lipid membranes is governed by three major forces: (i) hydrophobic repulsion between alcohol and water (strongly favoring membrane partitioning); (ii) a much weaker attraction of the polar group of the alcohol to the head group of the lipids, and (iii) a weak repulsive effect due to the intercalation of the alcohol acyl group into the lipid bilayer.

The composition of the lipid membrane strongly affects the thermodynamics of alcohols partitioning. Thus, 3 mol% C in a phospholipid membrane increases its propensity to adsorb alcohol, while C in high concentration (10–30%) strongly decreases it [85–90]. These changes can be rationalized from the structure properties of phospholipid-cholesterol mixtures as revealed by their phase diagram [90]. Also addition of small amount (10 mol%) of ceramides increases the partitioning of ethanol into fluid bilayer membranes by about a factor of 3.

Systematic variation of chemical structure of lipid “impurities” such as ceramides added to phospholipid membranes suggest that the “backbone”, not the hydrophilic head-group of the lipid is pivotal for the effect on alcohol partitioning [88–93].

3.3. Chain-length dependent effects of alcohols on the purple membrane of Halobacteria

The effects of alcohols from 1-butanol to 1-hexadecanol and fatty acids from hexanoic acid to hexadecanoic acid on the purple membrane of Halobacterium salinarum are under intense investigations [94]. The purple membrane contains a single protein, bacteriorhodopsin (BR), which binds a retinal chromophore and shows an absorption maximum at 560 nm (in the dark adapted state). Alcohols decrease the absorbance of BR with a small shift of its maximum to the shorter wavelength at the lower concentration and a large shift to about 480 nm at the higher concentration (Fig. 5). In the figure, two distinct isosbestic points are observed at 513 and 475 nm,
respectively, and the transition between two points occurs around 1.3 mM. It indicates that alcohols act on the purple membrane in two different manners depending on the concentration. Similar result was also reported for volatile anesthetics [95,96]. Fig. 6 shows the dependence of $C_{0.8}$ on the carbon chain lengths. Here, $C_{0.8}$ stands for the concentration where the absorbance of BR at 600 nm decreases to 80% of the original value. As shown in Fig. 6, the potencies of alcohols for the effect on the absorbance increase with their carbon chain length but level off after 1-tetradecanol (cut-off effect).

In contrast, the potencies of fatty acids increase with the carbon chain length between hexanoic acid and hexadecanoic acid. The so-called cut-off effect in anesthetic potency is observed generally and remains unresolved problem in anesthesia [97–99]. As the partition of alcohols into purple membrane is not known, it cannot be discussed about whether the observed cut-off in potencies is due to a corresponding cut-off in partitioning into membrane.

However, it is suggested by the occurrence of cut-off effect that the effect of alcohols on the purple membrane may be closely related to anesthesia.

4. Modification by local anesthetics of biomembrane structure and function

The subject if membrane lipid bilayer or the membrane proteins and receptors are the main molecular targets of local anesthetics is still on debate. To elucidate the great diversity of structural and functional modifications induced by local anesthetics at the membrane level, a large number of biophysical and biochemical approaches have been used. Our results in this field as well as recent literature data on this topic are briefly reviewed.

4.1. Structural effects of tertiary amine local anesthetics in model and biological membranes

Diffraction methods should be regarded as a first step towards an understanding of molecular mechanisms of interaction, which is supported by structural data [100].

For this reason we have used small- and wide-angle X-ray diffraction to study the influence of P and lidocaine on the structure of egg lecithin films. The measurements were performed with a Siemens Kristaloflex IV diffractometer, using therapeutical concentrations corresponding to samples of 0.014 and 0.14 mol drug/kg dry phospholipids. Our results indicated that both local anesthetics increased the mean distance between the hydrocarbon chains, even at therapeutic concentrations of the order of 10 nmol anesthetic/mol phospholipids [101,102]. When for example P was added, the peak decreased and broadened with the increase of drug concentration (Fig. 7).
Fig. 7. The evolution of the main crystalline peak of the gel phase with increasing P concentration in egg lecithin.

The peaks at high-angle X-ray scattering allowed to reveal the packing of lipid molecules. At the order–disorder transition temperature, the gel phase (ordered) transforms into the liquid–crystal phase. In the gel phase, the lipid gives rise to a strong two-dimensional hexagonal packing of the chains with a center-to-center separation of 4.85 Å [103].

Hydrocarbon chains in the liquid crystalline phase produce a broad diffuse peak of X-ray scattering at about 4.6 Å spacing [104]. The regular hexagonal bidimensional packing of the fatty acyl chains at low temperature may be perturbed by anesthetics. The perturbation of the egg lecithin layer inferred from our X-ray data is in good agreement with literature data [105,106] and suggests that local anesthetics molecules were partially intercalated into lipid layer.

Moreover, when egg lecithin bilayer were subject to the action of ultrasonic vibration, it was shown that P rendered the phospholipid membranes more resistant to degradation by ultrasound and stabilized the major lamellar phase [102].

Anesthetic partitioning into lipid bilayer membranes has been studied by various methods, e.g. equilibrium dialysis [107], ion-selective electrodes [108–110]. The differences in the partition coefficients to the resting and excited states of cationic P and lidocaine to liquid–crystalline and solid–gel DMPA membranes were correlated to the nerve blocking potencies and is supposed to be the cause of local anesthesia [111].

Differential scanning calorimetry (DSC) and fluorescence measurements were used to study the effects induced by dibucaine, tetracaine and P in skeletal muscle sarcoplasmic reticulum membranes. Millimolar concentrations of dibucaine and tetracaine were shown to lower by several degrees the denaturation temperature of the membrane (Ca²⁺, Mg²⁺)-ATPase, while P failed to produce such an effect up to a concentration of 10 mM. This result was in good correlation with the fact that the order parameter of the sarcoplasmic reticulum membrane lipids, as inferred from the polarization of diphenylhexatriene fluorescence, was no significantly altered by P at concentrations up to 10 mM [112]. The DSC was used also to study the Krafft phenomenon of some local anesthetics (as hydrochlorides, hydrobromides or hydroiodides): tetracaine, dibucaine, bupivacaine. It was found that the Krafft temperatures of these anesthetics were greatly dependent on the chemical structure of hydrophobic groups in the molecules, and on the negative halide counterions [11,113].

Nuclear magnetic resonance (NMR) was used to investigate the changes in water permeability of phospholipid bilayer vesicles by tetracaine at concentrations lower than 10 mM also permitting some structural information to be deduced. The results suggested that when lipid bilayers were in the fluid state, lipid membrane integrity was mainly controlled by electrical interactions exerted between the charged polar headgroups of phospholipids. The positively-charged tetracaine molecules partially neutralized these membrane negative surface charges, thus destabilizing membrane structure and lowering the barrier for water permeation through phospholipid bilayers [114].

4.2. Influence of local anesthetics on mechanical properties of bilayer lipid membranes and liposome membrane

In addition to known alteration in microscopic membrane parameters (microviscosity, the parameter of ordering, quadrupole splitting, conductivity) local anesthetics may induce changes in the lipid bilayer structure, which may become reflected in the macroscopic mechanical mem-
brane properties. For instance, the conformational changes in the lipid head group, indicated by 2H-NMR spectroscopy [115] may be reflected in the ordering of the inner hydrophobic region of the membrane. Since local anesthetics considerably influence the hydrophobic region of the membrane, results of the interaction may be effectively detected by measuring the membrane electrostriction. The electrostriction is induced by application of d.c. or a.c. voltage to the membrane (with relatively small amplitude $V \sim 10$–100 mV; usually bilayer lipid membranes (BLM) are used for this purpose). The voltage compress the membrane with a pressure $p = CsV^2/(2h)$, where $Cs$ is the membrane specific electrical capacity, $h$ is the thickness of membrane hydrophobic part and this is reflected in changes of the thickness. The ability of membrane to change its thickness is characterized by elasticity modulus in direction perpendicular to the membrane plane $E = -p/(Dh/h)$ [116]. The influence of lidocaine, carbisocaine and mesocaine (at concentration range 10 µM to 1 mM) on the membrane electrostriction was reported. It has been shown (i) that the ability of local anesthetics to decrease the elasticity modulus $E$ of BLM of various compositions (i.e. the compressibility of BLM increases) is stronger for local anesthetics with longer hydrophobic chain (carbisocaine) than for that with less expressed unpolar part (lidocaine) [117]; and (ii) that increase of concentration of neutral form is associated with more pronounced changes of elasticity modulus. The neutral form of tetracaine induced more pronounced changes of dipole potential of BLM in comparison with the charged form [118]. This effect was explained by means of deeper incorporation of neutral form into BLM, so more disturbing effect on the ordering of polar and hydrophobic part of the membrane might be expected. This conclusion was recently confirmed by measuring volume compressibility of liposomes by means of sound velocity method [116]. The neutral form of tetracaine induced larger increase of volume compressibility in comparison with charged form [119]. The interaction of mesocaine with BLM showed that this local anesthetic is also able to change membrane electrostriction; however, the effect depended on initial structural state of the BLM [117].

The generalized restructuring of the lipid bilayer, as reflected in the changes in the mechanical characteristics, has a significant influence on the operation of functional subunits e.g. ionic channels formed by gramicidin in BLM [120]. The results, at least those obtained for local anesthetics with large hydrophobic moieties, support the concept of non-specific interaction of local anesthetics with lipid bilayers, mediated by alterations of the structural state of the latter.

4.3. Red blood cell membranes and tertiary amine local anesthetics

As concerns the model, we considered that the red blood cell membrane is sufficiently simple and well described with respect to its molecular architecture to allow clear-cut conclusions. The biological relevance of the observed effects is more direct than in the case of reconstituted models.

The osmotic fragility is regarded as one of the best indices to describe the functionality of erythrocyte membranes, and the rate of hypotonic hemolysis measures the cell rupture process, thus conveying complementary information on membrane fluidity and mechanical properties. Early in 1972, it was suggested that P protected erythrocytes against osmotic hemolysis and increased membrane fluidity [6].

Our data on the temperature dependence of osmotic hemolysis in the range of 22–42°C revealed the existence of critical points in the antihemolytic effect of lidocaine; in this case, the antihemolytic effect reached a maximum at 37°C, which means that the antihemolytic effect was minimum at this temperature (Fig. 8) and then was followed by a minimum at 32°C.

The same kind of behavior was observed also with the other tertiary amines, but the temperature of the minimum antihemolytic effect was characteristic for each drug, being also 37°C for dibucaine, but 32°C for P, and 27°C for tetracaine. All these four local anesthetics are decreasing the rate of osmotic hemolysis suggesting a decrease of membrane fluidity, in contrast with the commonly held view. Thus, our data indicate the association of local anesthetics effects solely with the fluidizing action should not be taken for
This hypothesis gained more experimental support consisting of the investigation of rat erythrocytes by measurements of excimer fluorescence of their inter-molecular forming fluorophore 1,3-di(1-pyrenyl)propane. These results revealed that P decreased the fluidity of rat red cell membrane by 20% [124].

The positioning of P among the components of human erythrocyte membranes was investigated by electron paramagnetic resonance (EPR) using several spin labeled molecules: fatty acids, e.g. 5-, 12- and 16-doxyl-stearic acids, spin labeled P and a quaternary salt. It was inferred that P interacts with the membrane not only at the water interface, but also (at least) up to the fifth carbon atom of the hydrophobic tail of phospholipids [125, 126]. In order to verify whether P operates on certain sensitive membrane proteins, such as the ion channels that govern the response of nerve cell, the gramicidin-S was incorporated into dimiristoylphosphatidylcholine dispersion. The resulted two component EPR spectra of spin labeled SA (14-SASL) and phosphatidylserine (14-PSSL) display a selectivity towards the intramembranous part of the peptide [127].

Anesthetic molecules expand cell membranes. The phenomena show that the volume function and the cell shape with anesthetics in membranes are significant factors in molecular mechanisms of anesthesia [128].

Historically, Trudell [8], re-examining the data reported by Seeman [6], calculated that the volume increased to about equal the volume of incorporated molecules and criticized the Seeman hypothesis of excess volume increase. The excess volume may be created at any place in the total system; however this does not necessarily imply that the excess volume expansion is the direct cause of anesthesia.

Using the standard filterability test [129] and measuring the rate of erythrocyte packing in a centrifugal field [130], we reported that P increased the deformability of normal red blood cells, and partially restored the deformability of erythrocytes which had been previously hardened in vitro. This effect of P, which affects the volume function and the cell shape, was explained on the basis of its known ability to prevent intracellular accumulation of calcium ions in erythrocytes [131]. These results were similar to those obtained with more elaborated techniques such as EPR [132] and electron microscopy, which additionally revealed that P and tetracaine also changed erythrocyte morphology from the normal biconcave disk shape to stomatocytes [133].

Additional data were published on the influence of P hydrochloride on the aggregation, viscoelasticity, cell shape, volume, density and electrophoretic mobility of erythrocytes. The anti-aggregating effect of P hydrochloride in plasma is decreasing in time, probably due to the enzymatic hydrolysis of the drug.

Transmembrane water diffusion is an additional essential characteristic of the erythrocyte membrane functionality. The interaction of local anesthetics on cell membranes has been studied extensively in order to unravel its molecular mechanisms on whole organism [134]. We used a ¹H-NMR method based on the doping of erythrocyte samples with paramagnetic Mn²⁺ ions in order to investigate the dynamic membrane process, which occurs in a time interval of the order of milliseconds [135, 136]. Our NMR data revealed that P concentrations ranging from 1 to 10 mM increased the transmembrane water diffusional exchange in intact washed erythrocytes at 37°C by

![Fig. 8. Temperature dependence of the relative hemolysis in 65 mM NaCl buffered solution containing lidocaine in the concentrations indicated near each curve.](image-url)
20% compared to controls. This increase of the erythrocyte membrane permeability was linked to the conversion by low P concentrations of washed erythrocytes (i.e. crenated red cells) back to their normal discoid shape at constant cell volume. Subsequent increases of the transmembrane water exchange time (i.e. decreases of permeability) at P concentrations higher than 10 mM were only regarded as apparent and largely artifactual. These later modifications were ascribed to the important red cell swelling and the alteration of membrane structure, which are known to occur in this concentration range of the drug. Under the circumstances, at high drug concentrations the diffusion process could no longer be regarded as taking place at constant cell volume [137,138].

Although the functional significance of the phosphorylation of human erythrocyte membrane cytoskeletal proteins still remains elusive, the possibility has been considered that this process might be involved in mediating the interactions among some protein components of the membrane skeleton, thus maintaining membrane integrity and preventing membrane rigidity. Our investigations used 32P as a radioactive tracer for the quantitation of phosphorus enzymatic uptake into various membrane protein fractions [139]. The experimental data suggested that, at low concentration (2 mM), P acted as a moderate inhibitor of red blood cell protein kinase C-dependent phosphorylation of cytoskeletal proteins [140]. This protein phosphorylating enzyme, which now seems to be ubiquitous in mammalian cells is known to bind to Ca2+ ions, phorbol esters, diacylglycerol and to biomembranes and to phosphorylate protein substrate, thus altering critical cellular events.

4.4. Modification of the ion transport properties of biomembranes by tertiary amine local anesthetics

The results in the previous chapter indicate some structural change induced by tertiary amines in plasma membrane organization. It is to be expected that concomitant changes would occur in transport properties of the membranes. In an attempt to reveal such changes, we have investi-
The EPR technique using paramagnetic nitroxide stable free radicals as molecular probes was successfully used to measure the permeability of egg yolk lecithin liposomes to ascorbate ions [149] and hydrogen cations [150].

When in their cationic form, all tertiary amines investigated (i.e., P, lidocaine, dibucaine and tetracaine) were shown to increase the permeability of unilamellar lecithin membranes to ascorbate ions. The stimulating potency correlated well with the partition coefficients of these drugs in the lipid phase, and decreased in the following order: dibucaine > tetracaine > lidocaine > P. An explanation of this effect was attempted on the basis of two hypotheses. It was assumed that either the incorporation of charged drug molecules into the phospholipid bilayer created some structural defects, thus facilitating the passage of ascorbate ions, or the positively charged tertiary amine molecules distributed into the liposomes surface and attracted the negatively charged ascorbate ions, increasing the probability of their diffusion through the structural defects created in the lipid bilayer.

The uncharged drug molecules produced the opposite effect, i.e. decreased the permeability of liposomes to ascorbate. This was accounted by the fact that uncharged local anesthetics penetrate more deeply into lipid membranes, producing a tighter lipid packing thus inhibiting ascorbate diffusion through lipids [149].

The same four local anesthetics (dibucaine, tetracaine, lidocaine and P) were found to increase liposome permeability to hydrogen ions by forming transient pores into lipid barriers or by intercalating into the “proton conductive pathways”, which are postulated to exist in unilamellar lecithin liposomes. However, in this case, the order in which tertiary amines increased the proton permeability of liposomes was that of decreasing p\(K_a\) value (i.e. procaine \(pK_a = 8.9\), tetracaine \(pK_a = 8.4\), dibucaine \(pK_a = 8.0\) and lidocaine \(pK_a = 7.8\)). These results suggest that in order to cross the lecithin bilayers, protons may use a different conductive pathway, which is not available to other ions. The coincidence between the order of increasing proton permeability and that of decreasing of \(pK_a\) values for tertiary amines suggests that, when translocating from one side of the membrane to the other, proton can move along hydrogen bonds. Tertiary amines could intercalate into these “proton wires” thus contributing to the “conductance”, considering their proton donor properties. However, before extrapolating the implications of such a hypothesis of tertiary amines producing local “short circuits” in membranes to the molecular mechanisms of local anesthesia, these data should be confirmed also in the case of the influence of tertiary amines on the proton permeability of natural membranes [150].

Protein moderately reduced the true affinity of ATPase for ATP, whereas equilibrium binding of \(Ca^{2+}\) to ATPase in the absence of ATP remained virtually unmodified. The various intermediate steps in the ATPase catalytic cycle were investigated with fast-kinetics technique in order to identify those steps which were targets for inhibition by P. Procaine slowed down ATPase dephosphorylation which was at least a partial cause of the observed inhibition of overall ATPase activity. In contrast, P accelerated the calcium-induced transconformation of unphosphorylated ATPase in the absence of ATP and altered neither the rate of the \(Ca^{2+}\)-dependent phosphorylation of ATPase nor the rate of the dissociation of \(Ca^{2+}\) from phosphorylated ATPase towards the sarcoplasmic reticulum lumen [151].

In human erythrocyte membranes, P concentrations ranging from 0.1 to 1 mM were found to stimulate in a concentration-dependent manner the activity of \((Ca^{2+}, Mg^{2+})\) ATPase, but had no effect on the equivalent enzyme from smooth muscle sarcolemma [152].

5. Effects of procaine on platelet aggregation

Blood platelets play essential roles in both hemostasis and thrombosis through four important mechanisms: the interaction with the constituents of blood vessel walls, the secretion of platelet constituents, the adherence of platelets to each other (platelet aggregation) and the enhancement of fibrin formation and fibrin-linking.

Our studies in vitro regarding the influence of P on platelet aggregation revealed that this adher-
ence of platelets to each other induced by adenosine diphosphate (ADP), thrombine and epinephrine was inhibited by P. However, while rather high concentrations of P (10−20 mM) were required in order to inhibit ADP or thrombin-induced aggregation, a complete inhibition of epinephrine-induced aggregation was obtained with only 1 mM P. This result suggested that specific platelet receptor, while the effects on ADP- or thrombin-induced platelet-aggregation were ascribed to an unspecific membrane process [153]. Further studies investigated the platelet aggregation induced by a mixture containing sub-threshold concentration of ADP, epinephrine and collagen. Under these conditions, which were closer to those occurring in vivo, 0.2 mM P caused an 80% inhibition of platelet aggregation, while much higher concentrations of P were required to inhibit aggregation when each of the above-mentioned stimuli was used alone in sub-maximal amounts [154]. In the presence of lesser physiological concentrations of Ca^{2+} ions and lower amounts of plasma proteins, a 95–100% inhibition of epinephrine-induced platelet aggregation was obtained with P concentrations ranging from 25 to 100 µM [155].

On the basis of the experimental data, a possible interaction of P with the platelet 2-adrenergic receptors has been suggested. This explanation should also take into consideration the structural similarities existing between the molecules of P and epinephrine. However, the molecular details of such an interaction are still to be elucidated.

### 6. Theoretical modeling of the interactions between local anesthetics and biomembranes

Based on diffusion process of local anesthetics through the membrane system [156] or on a target theory description of the chemical inactivation of membrane function [157] we reported, by comparing with experiments, a new theory of relaxation for the interaction of chemical compounds with membranes [158]. Our theory allows us to characterize the nearly exponential laws of relaxation. It is fundamentally different from other theories in that it involves a hierarchy of parallel pathway with different time scale for the relaxation process. This physical mechanism is naturally described using a non-Archimedean (NA) formalism. Our starting point was the NA mathematical analysis as presented in the literature [159].

Firstly we used our NA model for the classification and grouping of 27 local anesthetic agents currently in use on the basis of their similarity with P. The model starts from the simple assumption that chemically similar molecules have similar pharmacological properties. Subsequently, the similarity indices and non-Archimedean distances were defined and justified, and different partition into classes of compounds were compared using an entropy defined as a criterion of imprecision in classification [160]. For the same purpose of establishing a good structure–activity correlation, a new topological index has been proposed, which describes well the drug activity for various classes of substances. This new parameter simultaneously accounts for both the molecular branching and the hydrogen content of the molecule [161].

A more complex model of drug action was developed on the basis of polystochastic methods [162]. This new model was capable of simulating some basic properties of living systems, such as the learning and the memory faculties, the pattern recognition ability and the control capacity. It was considered by its authors as a preliminary step in the process of mathematical modeling in the interactions between drugs and complex biosystems, with final relevance of the clinical practice and to potential anticipations of the biological actions of certain molecules.

A single-compartment [163] and multi-compartment model [158,164] have been developed to describe the relaxation of the action potential in nerve cells in the presence of P. These are actually multi-scale non-exponential models for the interaction between anesthetic molecules and biomembranes. They are based on the assumption that the drug–membrane interaction is a relaxation process resulting from superpositions of different component processes which take place at different scales of time. The changes of the whole process at slower time scale could be determined by changes at faster scales. An expression using La-
guerre polynomials was obtained as a best fit for experimental data. The theoretical predictions of the model were discussed with respect to the experimental presence of 10 mM P. In this particular case, the best correlation was obtained using a single-compartment model [158,164].

A computer simulation was performed on a simple but general molecular model of the interaction of both general and local anesthetics with lipid membranes. In the vicinity of the gel-to-fluid membrane phase transition it was found that anesthetics had a strong effect on the heterogeneity of the membrane and induced regions of locally high drug concentrations. This led to a broadening of the specific heat peak and a maximum in the membrane/water partition coefficient. The results were in good agreement with available experimental data [165]. The same authors proposed a more general microscopic interaction model to describe the changes in the physical properties of the phospholipid bilayer due to the presence of foreign molecules which partition between the membrane phases and the aqueous environment. This model showed a very good correlation with existing experimental data on local anesthetics like P and could be used in the case of volatile general anesthetics [166].

7. Conclusions

From our studies of interfacial phenomena, modification of biomembrane structure and function, lipid phase transition, fluidity of lipids and proteins, and membrane expansion, it has been inferred that interfacial properties and core properties of biomembranes are closely coupled. In other words, any change in interfacial properties alters core properties, and vice-versa, core properties affect interfacial properties. It can be considered that the interfacial properties are the primary parameters determining anesthetic effects. Within the membrane, P is capable of concentrating at either lipid–lipid or lipid–protein interfaces and can presumably affect the lipid environment of the sodium channel. While this general effect may well explain the ability to create anesthesia, it does not exclude the possibility that specific receptors for some local anesthetics may also be present.

The studies of molecular dynamics of lipid association at the hydrophobic interface of gramicidin-S done with spin-labeled molecules (e.g. P, etc.) using ERP technique as revealed in our preliminary results [127,167] are in principle convenient comparisons with theoretical dynamical models. This opens the way for detailed comparisons with the results of dynamical calculations and optical spectroscopic experiments.

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