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Biophysical Chemistry 80 (1999) 53–66

Biophysical Chemistry

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# Interaction of the local anaesthetic heptacaine with dipalmitoylphosphatidylcholine liposomes: A densimetric study

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Received 9 December 1998; received in revised form 23 April 1999; accepted 23 April 1999

**Abstract**

Interaction of the local anaesthetic heptacaine, monohydrochloride of [2-(heptyloxy)-phenyl]-2-(1-piperidinyl)-ethyl ester of carbamic acid, with multilamellar dipalmitoylphosphatidylcholine (DPPC) liposomes in aqueous solution with high excess of water has been studied by means of density measurements in the scanning regime in the main phase transition region. The anaesthetic decreased the temperature of main phase transition. The molar partition coefficients of heptacaine between aqueous phase, liquid crystal and gel phases of DPPC have been determined from a combination of phase transition data obtained by densimetry with a DPPC/heptacaine phase diagram published in the literature. The saturation of heptacaine concentration in liposomes has been observed at higher total amount of anaesthetic. The partial specific volume of heptacaine located in DPPC bilayers is slightly lower than in the aqueous phase. 

*Keywords:* Local anaesthetic; Dipalmitoylphosphatidylcholine; Density meter; Lipid bilayer; Phase transition; Partition coefficient

## 1. Introduction

Local anaesthetics can interact directly with membrane proteins as well as they can alter their function due to the modification of structure of surrounding lipid bilayer. To date, the role of these two mechanisms in final biological effect is not clear [1,2]. It has been shown in several papers, that the biological effect of anaesthetics correlates well with the decreasing of the transition temperature between solid-like (gel) phase and fluid-like (liquid crystalline) phase in model

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0301-4622/99/$ - see front matter 
PII: S0301-4622(99)00061-7

phospholipid membranes [3–9]. These findings indicate the importance of anaesthetic-phospholipid bilayer interactions in the whole process.

In the present paper we study effects of local anaesthetic heptacaine, monohydrochloride of [2-(heptyloxy)-phenyl]-2-(1-piperidinyl)-ethyl ester of carbamic acid, on the main (gel–liquid crystal) phase transition in multilamellar dipalmitoylphosphatidylcholine (DPPC) liposomes. Heptacaine is one of the most potent local anaesthetics prepared — in comparison to anaesthetic standard cocaine, its relative potency is 100-times higher in the surface anaesthesia; to procaine 170-times higher in the infiltration anaesthesia; and 94- and 98-times higher in the blocking of action potential on axons and isolated nerves, respectively, while the corresponding relative efficiencies of clinically used, commercially available and widely studied lidocaine are only 0.5, 2, 7.1 and 3.4, respectively [7]. Groups of Dörfler and Balgavý [10–19] studied interactions of heptacaine and of its alkyloxy homologues with various biological and model membranes using different experimental techniques, including their effects on phase transitions in DPPC. In contrast to differential scanning microcalorimetry (DSC) used by the quoted authors, in the present work we study phase transition effects by means of buoy density measurements. This method of specific volume determination is rigorously non-invasive, similar to DSC. However, it has one serious advantage — the scanning over the temperature range can be arbitrarily slow so that it allows the perfect equilibrium on macroscopic as well as on the microscopic levels. In the last decade, this method gave serious experimental data concerning the structure of model phospholipid membranes [20–26] and their interactions with different species [27–29].

In the lipid bilayer, heptacaine polar groups will interact with phospholipid polar head groups and its alkyloxy chain will orient parallel to the lipid hydrocarbon chains. It has been suggested, that due to this interaction, the bilayer should expand laterally, and the free volume in the hydrophobic core of the bilayer formed because of the mis-match between the lengths of lipid and heptacaine hydrophobic parts should be filled-in immediately after its formation by the *trans-gauche* hydrocarbon chain isomerization and/or by the chain interdigitation [12–19]. Supposing that the anaesthetic and lipid molecular volumes are additive in the bilayer, it has been deduced from diffraction studies that heptacaine causes a decrease in bilayer thickness [19]. On the other hand, it has been supposed in Monte-Carlo modelling studies of interactions of different molecular species with lipid membranes [2,30–34] that the anaesthetic molecules occupy interstitial positions between the acyl chains of lipid molecules. Implication of this idea is that the entropy of mixing is negligibly small and from it follows, that the incorporated anaesthetic molecules have effectively zero partial molar volume in bilayer [30,31]. Similar effect has been observed by King et al. [45] who found that hexane dissolved in fluid phosphatidylcholine bilayers has a partial molar volume of approximately 0 up to relatively high concentrations. The excess molar volume of hexane in bilayers is thus negative. This is in contradiction to earlier results [35–38]. Bull et al. [36] and Franks and Lieb [37] have reported, that the volume of volatile anaesthetics did not change when transferred from water to membrane (i.e. the excess volume is 0), while Kita and Miller [38] and Mori et al. [35] have found, that the total volume of the lipid suspension increases when anaesthetic molecules are transferred from aqueous phase to membrane (i.e. the excess volume is positive). The final aim of the present work is thus obtaining of the specific volumes of constituents in the heptacaine/DPPC/water system.

## 2. Materials and methods

### 2.1. Chemicals

Synthetic dipalmitoylphosphatidylcholine (DPPC) was obtained from Fluka and was used without any purification. Local anaesthetic heptacaine prepared as described by Čižmárik and Borovanský [39] was kindly provided by Professor J. Čižmárik of Faculty of Pharmacy, Comenius

University, Bratislava. Water was triply distilled, once from the alkaline potassium permanganate solution.

### 2.2. Preparation of samples

The aqueous solution with highest concentration of local anaesthetic was prepared at the beginning of the experiment. This starting solution was used for preparation of each sample with any concentration of local anaesthetic. Appropriate amount of this solution was added to $\sim 6.6$ mg of DPPC in a small glass flask and the final concentrations of sample was obtained by completion with degassed water to 2.6 ml. This sample was heated to 58°C and a homogeneous dispersion was obtained by intensive stirring during 10 min. After the following degassing the sample was filled into the measuring chamber in the buoy of density meter. The measuring started after 24 h stabilisation at 30°C. This procedure is compatible with that described in classical papers of Nagle and Wilkinson [23,24]. Our previous experiments have demonstrated, that for the system lipid–water the results are the same as obtained by the method described by Melchior et al. [27].

The weights of DPPC, heptacaine and the used solutions were measured on the Sartorius balances with an accuracy of $\sim 1 \times 10^{-4}$ g. Each obtained weight was corrected to the content of water in chemicals, the corrections were previously established by drying under vacuum.

### 2.3. Density meter and measuring

The density meter constructed and built in our laboratory as described by Bánó and Bán [40] was used for measuring the specific volumes. It is well suited for measuring the densities of small amounts of solid, solutes or dispersed materials in a liquid environment. The principal part of our density meter is a glass buoy (Fig. 1), which is immersed in water and is balanced by the electromagnetic force. The upper closed part of buoy is empty, in the lower part is a chamber for the measured sample. This chamber is closed by a glass stopper. Taking into account, that on microscopic level the contact between the glass surfaces of the buoy and stopper is not ideal, it is evident, that this closure is not absolute. The liquid remains in the gap between glass parts and thus it will flow out or in if the pressure in sample is not in equilibrium with the pressure in outer water. This fact is very important for the correct working of our density meter.

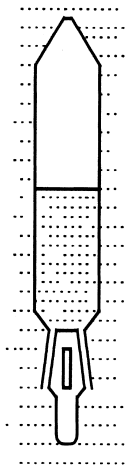

Fig. 1. Scheme of the glass buoy used in the density meter. The buoy is equilibrated by electromagnetic force in water. The chamber with sample is located in the lower part of buoy. This chamber is closed with glass stopper containing a small permanent magnet.

The equilibrium position of the buoy is checked optically. The balancing electromagnetic force acts to the permanent magnet located in the stopper. In the scanning regime (the temperature range 30–45°C, the scanning rate 4 mK/m), the above mentioned slow flowing of the solvent around the stopper appears. It causes the loss of solvent — approximately 0.5%, totally, in our temperature range. The 'base line' obtained in independent measuring scan of water sample with the same scanning rate is used for the comparison with measured data with the aim to avoid the shift caused by the effect described above as well as by more tiny effects. Nevertheless, also in this case, the upper mentioned effect generates the error of measuring, if the concentration of solution is non-zero. This error has been analysed in our previous paper [40] and in the presented experiments it was less than the sensitivity of measuring. The accuracy of measuring of the

equilibrium force is $1.5 \times 10^{-8}$ N. This means, that the liquid density is measured with the accuracy $\approx 1$ mg/l in the 1.47-ml chamber, the sensitivity of temperature measuring is 1 mK. The reproducibility of device was checked many times by the measuring of different solutions and dispersants. For the thermal expansion of DPPC liposomes in a water environment ($\sim 6.6$ mg/ml) it is better than 0.13 μN. The reproducibility is thus given by the reproducibility of sample preparation.

Fig. 2 shows schematically the principles of the experiments performed. In experiment Fig. 2a, the buoy is filled-in by water and balanced by the electromagnetic force $F_0$. The capillary in the bottom of the buoy in Fig. 2 illustrates symbolically the gap between the main body of the buoy and the stopper. At the change of temperature, the water flows around the stopper, i.e. through the capillary in the schematic diagram in Fig. 1. The temperature dependence of the force $F_0$ is used to calculate the 'base line' for the other experiments.

In experiment Fig. 2b, the buoy is filled-in by the aqueous solution of heptacaine and the buoy is balanced by the electromagnetic force $F_a$. In this experiment, we have studied the temperature and concentration dependence of the apparent specific volume of heptacaine in the aqueous phase. The results of this experiment were described and analysed in detail in our previous work [41]. The results of this experiment will be used below.

In experiment Fig. 2c, the buoy is filled-in by the aqueous dispersion of DPPC multilamellar liposomes and balanced by the force $F_p$. The results of this experiment were described and analysed in our paper [40], we shall also use them in the present paper below.

Finally, we show three hypothetical models Fig. 2d, e and f, used for comparison with data obtained for heptacaine solution and DPPC dispersion mixed in the buoy. If there were no partition of heptacaine in the DPPC bilayers (Fig. 2d), the number of anaesthetic moles in the DPPC liquid crystal and gel phases would be $N_{af} = 0$ and $N_{ag} = 0$, respectively, and the electromagnetic force $F_{p,a}^{(0)}$ needed to balance the buoy would be

$$F_{p,a}^{(0)} = F_a + M_p \cdot g(\rho'_a v_p - 1), \tag{1}$$

where $M_p$ is the mass of DPPC in the buoy, $g$ is the gravitation constant, $\rho'_a$ is the density of the anaesthetic solution and $v_p$ is the specific volume of DPPC. It is easy to show that the specific volume of DPPC is then

$$v_p = \frac{M_p + \Delta F_{p,a}/g}{\rho'_a . M_p}, \tag{2}$$

where $\Delta F_{p,a} = F_{p,a}^{(0)} - F_a$. In case of heptacaine partition into the DPPC bilayer, a part of anaes-

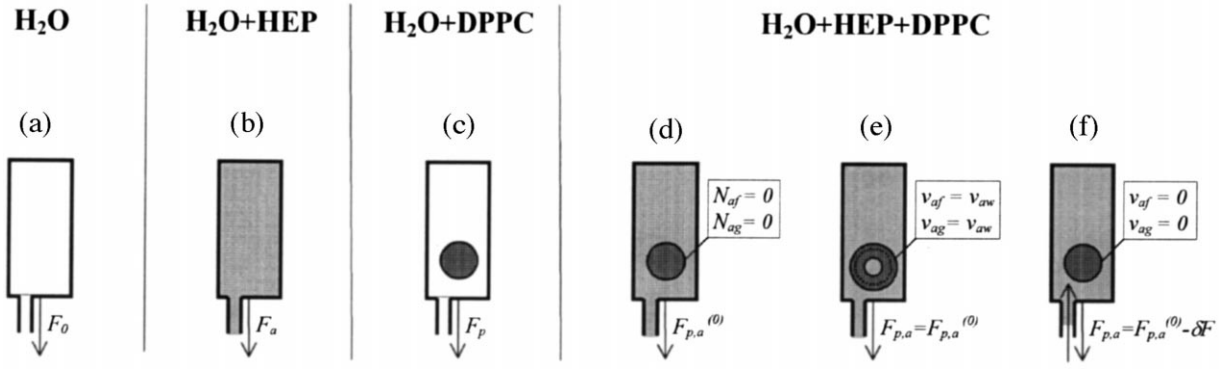


Fig. 2. Schemes of different experimental situations. The parallelograms symbolise the experimental chamber in buoy, the capillary in its lower part is a symbol of gap between the surfaces of stopper and body of buoy. For more details see text.

thetic molecules is located inside the bilayer. The Fig. 2e and f depict two different extreme possibilities. In the Fig. 2e, it is supposed that the volumes of DPPC and anaesthetic in the bilayer are additive, i.e. the specific volume of anaesthetic located in the bilayer is the same as in the aqueous phase. It can be written then $v_{af} = v_{aw}$ and $v_{ag} = v_{aw}$, where $v_{af}$, $v_{ag}$ and $v_{aw}$ are the specific volumes of anaesthetic in the liquid crystal DPPC bilayer, in the gel DPPC bilayer and in the aqueous phase, respectively. The whole volume of the sample in buoy will be the same as in Fig. 2d and the experimental result for this situation will be $F_{p,a} = F_{p,a}^{(0)}$. In this case the specific volume of the phospholipid part of the DPPC bilayer can be calculated by using Eq. (2) where

$$\Delta F_{p,a} = F_{p,a} - F_a \tag{3}$$

In Fig. 2f, it is supposed that specific volume of the anaesthetic located in the DPPC bilayer is 0, i.e. $v_{af} = 0$ and $v_{ag} = 0$. In this case the volume of the bilayer will be constant, but the whole volume of the sample will be decreased. The missing volume will be equilibrated by an inflow of water inside the capillary. The result is that the weight of the buoy is higher and the force needed to balance the buoy is

$$F_{p,a} = F_{p,a}^{(0)} - \delta F \tag{4}$$

The difference of forces $\delta F$ is obtained by using the relation

$$\delta F = g \cdot N_a(0) \cdot V_{aw} \cdot \rho_a' \cdot V_k \tag{5}$$

where $N_a(0)$ is the number of moles of anaesthetic with the zero volume in 1 ml of the sample, $V_{aw}$ is the molar volume of the anaesthetic in aqueous solution, $\rho_a'$ the density of the anaesthetic solution and $V_k$ is the total volume of the sample.

The situation in the Fig. 2d is only hypothetical, because it is well known that heptacaine does partition into DPPC bilayers both in the liquid crystal and gel phases [16]. No one of the two remaining possibilities Fig. 2e and f, or their combination can be excluded a priori. The value of $F_{p,a}^{(0)}$ cannot be obtained directly in the experiment, though it would be needed for the evaluation of results and discrimination between the possibilities Fig. 2e and f. Fortunately, the force $F_{p,a}^{(0)}$ can be calculated with a reasonable accuracy from the experiments Fig. 2a, b and c. It holds for the force $F_p$

$$F_p = F_0 + M_p \cdot g(\rho_0 \cdot v_p - 1), \tag{6}$$

where $\rho_0$ is the density of water. It can be shown that in our experimental set-up

$$F_{p,a}^{(0)} - F_a \approx F_p - F_0 \tag{7}$$

with the precision better than $10^{-8}$ N. The conclusion is that within this precision we can discriminate between Fig. 2e and f (or between e and combination of e and f) in our experiments.

It is evident from the analysis above, that the experimentally determined force $F_{p,a}$ could be used for the estimation of the lipid and anaesthetic specific volume in the bilayer, if the partition coefficients of heptacaine between the aqueous phase and lipid bilayer were known.

## 3. Results and discussion

The first goal of our experiments was the determination of phase transition temperatures at different heptacaine concentrations in the sample, followed by the partition coefficient estimation from available data. Fig. 3 shows examples of the dependence of measured difference of electromagnetic force $\Delta F_{p,a} = F_{p,a} - F_a$ as a function of temperature for selected values of the molar ratio $X_{am}$

$$X_{am} = \frac{N_a}{N_p} \tag{8}$$

where $N_a$ is the number of moles of anaesthetic and $N_p$ the number of moles of lipid in the sample. The number of moles of DPPC is $N_p = 3.4$ mmol/l for each sample. The results obtained for pure DPPC ($X_{am} = 0$) show sharp changes of $F_{p,a}$ at the temperature of main phase transition ($T_m$

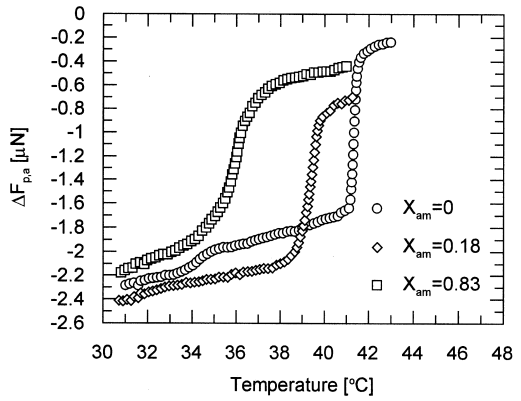


Fig. 3. The examples of measured electromagnetic force $\Delta F_{p,a}$ as a function of temperature at different concentrations of heptacaine in the sample. $X_{am}$ is the molar ratio between the anaesthetics and lipid in the whole sample. The scanning rate was 4 mK/m, number of moles of DPPC is $N_p = 3.4$ mmol/l for each sample. Only non-overlapping experimental points are shown.

$= 41.4$°C) and at the temperature of pretransition (34.3°C). These temperatures and half width of main phase transition ($\Delta T_{1/2} = 120$ mK) were obtained by the method described in paper of Nagle and Wilkinson [23]. The above presented temperatures of phase transitions are in good agreement with results published by other workers [20,23,27]. It should be pointed out that the phase transitions observed in the present work are sharper than those observed in most DSC experiments. This is due to the higher scanning rate used in DSC which causes the broadening of phase transition [42].

The addition of local anaesthetic at $X_{am} = 0.18$ causes the decreasing of the temperatures of both phase transitions and the broadening of their temperature span. At higher concentrations of the local anaesthetic, the pretransition is not detectable in our temperature range and the main phase transition is shifted further toward lower temperatures.

In the first step in the data evaluation, the dependence of temperature of main phase transition on the concentration of local anaesthetics was obtained. While the method of data analysis presented by Nagle and Wilkinson [23] is well suitable for pure DPPC without anaesthetic, for obtaining the phase transition temperature in samples containing the local anaesthetic, we have developed the more appropriate method using the relations published by Suezaki et al. [9]. According to these authors, the difference between the actual temperature $T$ in the region of main phase transition and the temperature $T_m$ of main phase transition of pure lipid, $\Delta T_m = T - T_m$, can be written as

$$\Delta T_m = -\frac{RT_m^2}{\Delta H}(1-\kappa)X_{am}\frac{1}{\kappa + \gamma(1-\kappa) + \dfrac{1}{P_{af}\cdot X_p}} \tag{9}$$

where $\Delta H$ is the enthalpy of main phase transition, $\kappa$ is the partition coefficient of anaesthetic distribution between the gel and fluid (liquid–crystalline) phases, $\gamma$ is the melted fraction of the membrane, $P_{af}$ is the partition coefficient of anaesthetic distribution between the liquid–crystalline phase and water and $X_p = N_p/N_w$ is the ratio between the number of moles of lipid $N_p$ and the number of moles of water $N_w$ in the sample.

The partition coefficients $P_{af}$ and $\kappa$ in the region of phase transition were defined by Suezaki et al. [9] by the relations

$$P_{af} = \frac{N_{af}}{N_{pf}} \Big/ \frac{N_{aw}}{N_w}; \tag{10}$$

$$\kappa = \frac{N_{ag}}{N_{pg}} \Big/ \frac{N_{af}}{N_{pf}}; \tag{11}$$

where $N_{af}$ ($N_{ag}$) is number of moles of anaesthetic in membrane in fluid (gel) phase, $N_{pf}$ ($N_{pg}$) is number of moles of lipid in fluid (gel) state and $N_{aw}$ is number of moles of anaesthetic in water.

The usually used definitions of partition coefficients [14,16,43] are

$$K_{p,fw} = \frac{N_{af}}{N_{af} + N_{pf}} \Big/ \frac{N_{aw}}{N_{aw} + N_w}; \tag{12}$$

$$K_{p,gw} = \frac{N_{ag}}{N_{ag} + N_{pg}} / \frac{N_{aw}}{N_{aw} + N_w}; \quad (13)$$

$$K_{p,gf} = \frac{N_{ag}}{N_{ag} + N_{pg}} / \frac{N_{af}}{N_{af} + N_{pf}} = \frac{K_{p,gw}}{K_{p,fw}}; \quad (14)$$

where $K_{p,fw}$ ($K_{p,gw}$) is the partition coefficient of anaesthetic distribution between the liquid–crystalline (gel) phase and water and $K_{p,gf}$ is the partition coefficient of anaesthetic distribution between the gel and liquid–crystalline phases.

The transformation of partition coefficients according to Eqs. (10) and (11) to that defined according to Eqs. (12)–(14) is simple only in the case when $N_{af} \ll N_{pf}$, $N_{ag} \ll N_{pg}$ and $N_a \ll N_w$. Under these conditions

$$P_{af} \cong K_{p,fw} \quad (15)$$

$$\kappa \cong K_{p,gf} \quad (16)$$

but in general this transformation depends also on the amount of lipid and anaesthetic in the sample.

In the following we will use the relation Eq. (9) for the estimation of the beginning and the end of phase transition. The melted fraction $\gamma$ in the region of phase transition can be expressed by neglecting the volume of phase boundaries between the gel and liquid–crystalline phases as

$$\gamma = \frac{v_p - v_p^g}{v_p^f - v_p^g} \quad (17)$$

where $v_p^g$ is lipid volume in the gel (solid) state and $v_p^f$ is lipid volume in the fluid state. The relation Eq. (17) can be rewritten as

$$\gamma = \frac{\Delta F_{p,a}^{pt} - \Delta F_{p,a}^{g}}{\Delta F_{p,a}^{f} - \Delta F_{p,a}^{g}} \quad (18)$$

where $\Delta F_{p,a}^{pt}$ is the measured difference of electromagnetic forces (according to relation Eq. (3)) in the region of main phase transition, $\Delta F_{p,a}^{g}$ is the measured difference of electromagnetic forces in the gel phase region and $\Delta F_{p,a}^{f}$ is the measured difference of electromagnetic forces in the liquid–crystalline region. The values $\Delta F_{p,a}^{g}$ and $\Delta F_{p,a}^{f}$ can be estimated in the region of main phase transition by a linear extrapolation of the $\Delta F_{p,a}$ measured at temperatures under and over this region, respectively. The relation Eq. (18) is valid if $\delta F = 0$ or if most of anaesthetic molecules are located in the aqueous phase (low lipid concentration). For higher lipid concentration and nonzero $\delta F$ the relation Eq. (18) is only approximate.

From the relations Eqs. (9) and (18) it can be obtained

$$\frac{1}{\Delta T_m} = -\frac{\Delta H}{RT_m^2} \frac{\kappa + \frac{1}{P_{af} X_p} + (1-\kappa)\frac{\Delta F_{p,a}^{pt} - \Delta F_{p,a}^{g}}{\Delta F_{p,a}^{f} - \Delta F_{p,a}^{g}}}{(1-\kappa) X_{am}}, \quad (19)$$

thus the value of $1/\Delta T_m$ is a linear function of the difference of forces $\Delta F_{p,a}^{pt}$ in the region of main phase transition.

On the Fig. 4 we demonstrate our method of data evaluation for $X_{am} > 0$, which follows from the application of expression Eq. (19). The value $\Delta F_{p,a}$ for the given anaesthetic concentration is presented as a function of $1/(T_m - T)$, where $T_m$ was found according to Nagle and Wilkinson [23]. The difference of forces is approximated by a linear function in the region of the phase transition as well as under and over it, respectively. These linear functions intersect in the points B and E indicating the estimated temperatures $T_B$ and $T_E$ of the beginning B and end E of phase transition, respectively. Finally, the phase transition temperature is obtained as $T_c = (T_B + T_E)/2$.

It can be seen that in the neighbourhood of B and E the real course of data cannot be described by this simple model, and that the position of B and E points is to a certain degree arbitrary. This behaviour can be understood from computer simulation of anaesthetic/lipid interactions [31], which shows that the crossing of solidus and liquidus curves in the phase diagram is not accompanied with sharp change of direction of tra-

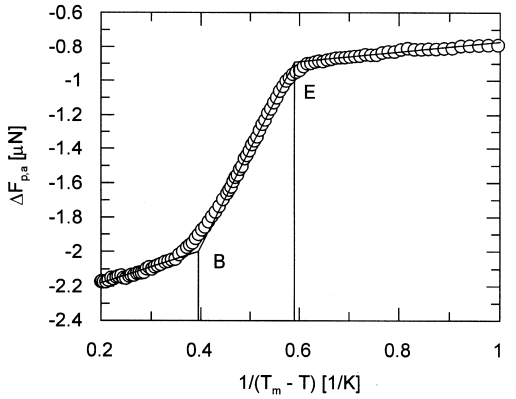


Fig. 4. Illustration of method used for the estimation of the beginning B and the end E of phase transition (for DPPC/heptacaine/water, $X_{am} = 0.18$).

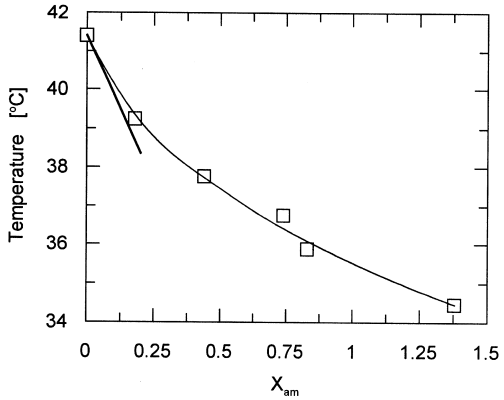


Fig. 5. Temperature of the main phase transition as a function of heptacaine content for DPPC/heptacaine/water samples. $X_{am}$ is the molar ratio between anaesthetic and lipid in the whole sample. The short thick abscissa shows the direction of curve for small $X_{am}$ according to Gallová et al. [14].

jectory like it is supposed in the model of the Suezaki et al. [9]. Nevertheless, the presented procedure allows to obtain the parameters of phase transition from experiment with relatively small uncertainty.

Using the described method, the shift of temperatures $\Delta T_B = T_m - T_B$ and $\Delta T_E = T_m - T_E$ of beginning and end of phase transition were found for each concentration of anaesthetic (see Table 1). In the Fig. 5, the temperature of phase transition $T_c$ is shown as a function of anaesthetic concentration. The short thick line for $X_{am} < 0.2$ show the dependence obtained by DSC at small anaesthetic concentrations by Gallová et al. [14,16]. It can be seen, that the calorimetric data agree well with the direction of our curve at $X_{am} \to 0$. Moreover, the anaesthetic depresses the temperature of phase transition less efficiently at higher concentrations. This indicates that in the lipid/anaesthetic/water system we have observed a saturation of anaesthetic effect.

To obtain the partition coefficients, we have further analysed our data in combination with the phase diagram published by Dörfler et al. [11]. This phase diagram is based on the DSC measurements of DPPC/heptacaine dispersion in 50 wt.% water. It follows from the theoretical analysis based on the paper of Suezaki et al. [9], that in samples with so small amounts of water nearly all anaesthetic molecules are located in the lipid bilayer. Moreover, the analysis of Chernik [44] and others (for references see [44]) shows, that

Table 1
Basic experimental results for different concentrations of local anaesthetic[a]

| $X_{am}$ | $\Delta T_B$ (K) | $\Delta T_E$ (K) | $K_{p,fw}$ | $K_{p,gw}$ | $K_{p,gf}$ | $\Delta H$ (kJ/mol) |
|---|---|---|---|---|---|---|
| 0.18 | 2.60 | 1.69 | $(1.72 \pm 0.74)\ 10^4$ | $(2.42 \pm 0.60)\ 10^3$ | $0.141 \pm 0.061$ | $38.6 \pm 9.5$ |
| 0.44 | 4.23 | 3.07 | $(7.29 \pm 1.08)\ 10^3$ | $(1.51 \pm 0.22)\ 10^3$ | $0.207 \pm 0.043$ | $28.9 \pm 4.2$ |
| 0.74 | 5.14 | 4.16 | $(4.50 \pm 0.48)\ 10^3$ | $(1.06 \pm 0.13)\ 10^3$ | $0.236 \pm 0.037$ | $24.2 \pm 2.9$ |
| 0.83 | 6.06 | 5.00 | $(4.53 \pm 0.45)\ 10^3$ | $(1.12 \pm 0.13)\ 10^3$ | $0.246 \pm 0.037$ | $22.3 \pm 2.6$ |
| 1.38 | 7.30 | 6.57 | $(2.96 \pm 0.19)\ 10^3$ | $(7.91 \pm 0.54)\ 10^2$ | $0.267 \pm 0.025$ | $19.4 \pm 1.6$ |

[a] $X_{am}$ is the molar ratio between the heptacaine and DPPC in the whole sample, $\Delta T_B = T_m - T_B$ and $\Delta T_E = T_m - T_E$ are the temperature shifts of beginning and end of main phase transition, respectively, $K_{p,fw}$ is the partition coefficient of heptacaine distribution between the DPPC in fluid state and water, $K_{p,gw}$ is the partition coefficient of heptacaine distribution between the DPPC in gel state and water, $K_{p,gf}$ is the partition coefficient of heptacaine distribution between the gel and fluid phases of DPPC and $\Delta H$ is the enthalpy of phase transition computed according to Eq. (20).

the phase properties of DPPC bilayer do not depend on the water content if it is higher than 25 wt.%. It can be supposed with a reasonable probability, that also the structure of DPPC/heptacaine system does not depend on water content over 50 wt.% and that the Dörfler's phase diagram describes the real content of anaesthetics in membrane also at high water excess.

To illustrate our approach, a part of this phase diagram is presented in Fig. 6 for the temperature region of $T > T_m - 7.5^{\circ}C$. On this phase diagram, we have found the points of the beginning ($B_i$) and the points of the end ($E_i$) of phase transitions for all anaesthetic concentrations used in the present work. Points of the beginning of phase transformation are located on the solidus and they were found according to their temperature $T_B$. The temperature $T_E$ allows to find the end of phase transition on liquidus. The trajectories of sample heating in phase diagram were obtained in agreement with ideas of Suezaki et al. [9]. Finally, the concentrations of the anaesthetic in the gel and fluid phases were directly obtained

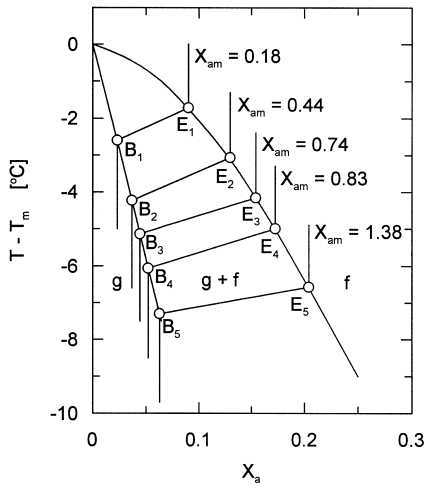


Fig. 6. Phase diagram for the pseudo-binary system DPPC/heptacaine in water with the trajectories of main phase transition for samples with different content of anaesthetic. $X_a$ is the molar fraction of the anaesthetic in the lipid membrane, $X_{am}$ is the molar ratio between the anaesthetic and lipid in the whole sample, $B_i$ and $E_i$ is the beginning and the end of phase transitions of ith sample. The liquidus and solidus curves were drawn according to Dörfler et al. [11].

from the $X_a$ coordinates of beginning and the end of phase transition. After a simple calculation, also the concentrations of anaesthetic in water as well as the molar fractions were obtained. It is supposed, that partition coefficients are constant over the phase transition, so the $K_{p,fw}$ can be simply computed according to Eq. (12) in points $E_i$ on liquidus curve, where all lipid is in the fluid state. Similarly, $K_{p,gw}$ can be computed in points $B_i$ on solidus. The obtained results are presented in Table 1.

From the relation Eq. (9) it can be derived the expression for the enthalpy of phase transition

$$\Delta H = -\frac{RT_m^2}{\Delta T_E}(1-\kappa)X_{am}\cdot\frac{1}{1+\dfrac{1}{P_{af}\cdot X_p}}. \qquad (20)$$

In the Fig. 7, the enthalpies at different anaesthetic concentrations obtained according to Eqs. (15), (16) and (20) are compared with enthalpies measured by Gallová et al. [16] using DSC. The experimental error of these DSC data is approximately $\pm 3.7\%$ (see Fig. 4. in the paper [16]). The errors of our data were computed from the experimental errors of boundaries between different phases in the used phase diagram given in [11]. The errors originating in the measuring of $T_B$ and $T_E$ are negligible in comparison with these errors. Taking into account these experimental errors and the approximate nature of the used equations, the difference between the DSC data and data obtained by using Eq. (20) ($\sim 17\%$ at $X_{am} = 0.18$) is rather small. The experimental errors of $\Delta H$ could be improved by new experiments giving better accuracy of phase diagram.

The obtained partition coefficients $K_{p,fw}$ were compared with the data published by Balgavý et al. [13] who studied the interaction of heptacaine and its alkyloxy homologues with unilamellar egg yolk phosphatidylcholine liposomes in fluid state by means of ultraviolet differential spectroscopy. As can be seen in Fig. 8, the value published for egg yolk phosphatidylcholine is in satisfactory agreement with our results. The partition coefficients, shown in Fig. 8, display a significant decreasing tendency with increasing anaesthetic

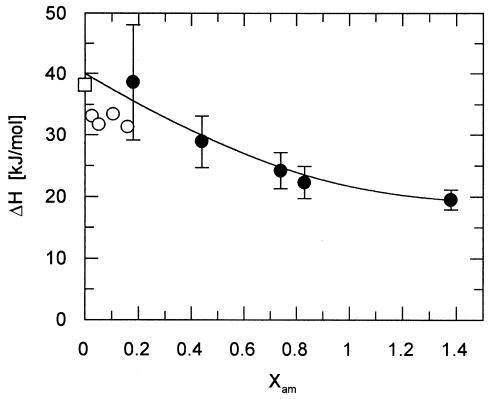


Fig. 7. The enthalpy of main phase transition computed according to Eqs. (15), (16) and (20) as a function of heptacaine content. $X_{am}$ is the molar ratio between the anaesthetics and lipid in the whole sample. The empty circles show the DSC data of Gallová et al. [16], the empty square is from the paper of Dörfler et al. [11].

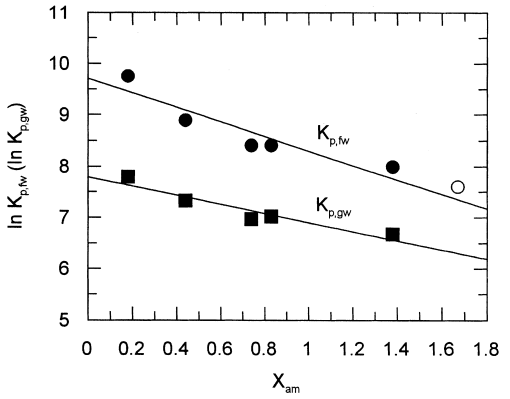


Fig. 8. The logarithm of partition coefficients of heptacaine between the fluid ($K_{p,fw}$) or gel ($K_{p,gw}$) phases of DPPC and water as a function of anaesthetic content. $X_{am}$ is the molar ratio between the anaesthetics and lipid in the whole sample. The empty circle shows the result of Balgavý et al. [13] obtained for the system egg yolk phosphatidylcholine/water in fluid state.

concentration demonstrating the saturation effect mentioned above. This saturation is more pronounced in the plot of molar fractions of anaesthetic in lipid vs. anaesthetic molar fraction dissolved in water (Fig. 9). This saturation is not the consequence of the formation of anaesthetic micelles in water, since the maximum heptacaine concentration in water used is only $\sim 64\%$ of its critical micelle concentration [18]. It seems that the saturation can be caused only by the mechanism connected with binding energy of anaesthetic molecule in the fluid model membrane. We suppose that the absolute value of the binding energy of anaesthetic molecule is lower in the neighbourhood of other anaesthetic molecules.

We should like to point out, that the procedure for obtaining the partition coefficients used in the present work can be applied in any case, when two experiments have been done — one with low water content and one with high. The used methods must be able to detect the phase transition — its beginning and end. From the experiment with low water content it can be obtained the phase diagram in the region of main phase transition, and in combination with the experiment with high water excess the partition coefficients can be derived. The 'low' or 'high' water contents should be judged according to the analysis presented by Suezaki et al. [9].

After having obtained the partition coefficients, the volume of anaesthetic molecules incorporated into the membrane can be analysed. The comparison of the DPPC specific volume in the absence and in the presence of heptacaine in the bilayers should be done at a comparable physical state of the bilayer, e.g. at a constant absolute temperature at which the bilayers are in the same phase

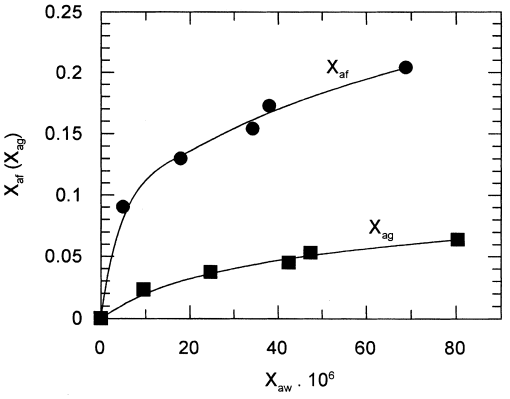


Fig. 9. Molar fraction of heptacaine in fluid $X_{af}$ or gel $X_{ag}$ phases of bilayer vs. the molar fraction $X_{aw}$ of heptacaine in water.

(gel or liquid-crystalline) or at a constant reduced temperature $T_r = (T - T_c)/T_c$, where $T_c$ is the main gel–liquid crystal phase transition temperature of the heptacaine + DPPC system. We have done this analysis at two reduced temperatures $T_r -$ at $T_r = -0.01$ and $T_r = 0.005$ for the gel and liquid–crystalline phase, respectively. In this analysis, it is convenient to use the value of difference $\Delta F_{p,a}$ as determined by using Eq. (3). Using Eqs. (4), (5) and (7), it can be shown that for the situation f in Fig. 2 (anaesthetic volume is 0 when located in the bilayer) this difference is (with the precision up to $1.10^{-8}$ N) given by

$$\Delta F_{p,a} = \Delta F_{p,0} - g \cdot N_a(0) \cdot V_{aw} \cdot \rho'_a \cdot V_k \qquad (21)$$

where $\Delta F_{p,0} = F_p - F_0$, and $N_a(0) = N_{ag}$ and $N_a(0) = N_{af}$ for the gel phase and liquid crystalline phase, respectively. It is evident that after substitution $N_a(0) = 0$, the Eq. (21) can be used also for the situation e in Fig. 2 (anaesthetic and phospholipid volumes are additive in the bilayer).

The calculated dependencies of $\Delta F_{p,a}$ are shown as a function of the molar ratio $X_{am}$ of heptacaine: DPPC in the whole sample and compared with the experimental data in Figs. 10 and 11 for the liquid–crystalline and gel phase, respectively. It is evident, that the experimental values are closer to the dependencies calculated by using the model which supposes the additivity of the anaesthetic and phospholipid volumes in the bilayer. However, there is a small but reproducible shift of the experimental data in comparison to the dependencies calculated by using this model. This shift does not depend within the experimental error on the anaesthetic concentration used. The final conclusion is that the partial specific volume of the anaesthetic is smaller when located in the bilayer than when located in the aqueous phase, i.e. the excess molar volume of heptacaine is negative. As described in the Introduction (Section 1), the excess volume of other anaesthetics in model and biological membranes has been observed to be zero [36,37] or positive [35,38]. This seems to be in conflict with our results. The positive excess volume is usually observed because the partial molar volume of anaesthetic in water is smaller than its molar volume, e.g. the volume of a methylene group of 1-alkanols is 15.9 ml/mol and 16.7 ml/mol in water and in pure alkanol liquid, respectively [38]. However, the partial molar volume of alkanols in model and biological membranes is close to their molar volume (in alkanol liquid) [38]. It is well known that the excess volume depends critically on solute–solvent intermolecular interactions — e.g. alkanols exhibit negative excess volumes in water and in sodium dodecanoate micelles but positive excess volumes in cyclohexane and *n*-heptane (see [38] and references therein). In case of heptacaine the partial molar volume in water is within the experimental error the same as in the solid state (in monocrystals) but smaller than in the heptacaine micelles or premicellar aggregates, i.e. heptacaine exhibits positive excess volume in this case [41]. Another very important factor which could have an impact on the experimental results is the anaesthetic concentration range studied. Similarly to our results in Figs. 10 and 11, the excess volumes of anaesthetics in membranes have been observed to be constant at relatively high anaesthetic concentrations in membranes in most papers. For example, the anaesthetic:lipid molar ratio range in the egg yolk phosphatidylcholine bilayers used in [38] was about 0.02:1–0.8:1 and 8:1–80:1 (!!) in case of 1-octanol and benzyl alcohol, respectively (see Fig. 2 in [38]). However, deviations from this constant values could occur at lower concentrations. For example, Mori et al. [35] have claimed that the total volume of the lipid suspension increases when the volatile anaesthetic enflurane is transferred from aqueous phase to membrane (i.e. that the excess volume is positive), but a closer inspection of their data (see first experimental point in Fig. 7 in [35]) shows the negative excess volume at low concentrations of enflurane. Another example is the seemingly contradictional finding of King et al. [45] who have observed that the partial molar volume of hexane in bilayers is 0 up to relatively high hexane:lipid molar ratio of 1:3. They have suggested that this effect is caused by filling-in defects (voids) in the lipid bilayer by hexane. Let us suppose that this mechanism operates not only in case of hexane, but also in case of other anaesthetics including heptacaine and that the negative excess

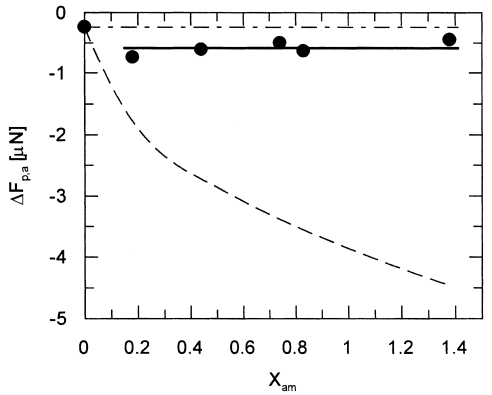


Fig. 10. Difference of forces $\Delta F_{p,a}$ obtained using Eq. (3) for the fluid (liquid–crystalline) phase at the reduced temperature $T_r = 0.005$ as a function of the anaesthetic:phospholipid molar ratio in the whole sample. Dotted-and-dashed line and dashed curve are the dependencies obtained by using Eq. (21) for the situation e in Fig. 2 (additivity of anaesthetic and phospholipid volumes in the bilayer) and f in Fig. 2 (zero anaesthetic volume when located in the bilayer), respectively, full line — mean value of experimental data for $X_{am} > 0$, circles — experimental data.

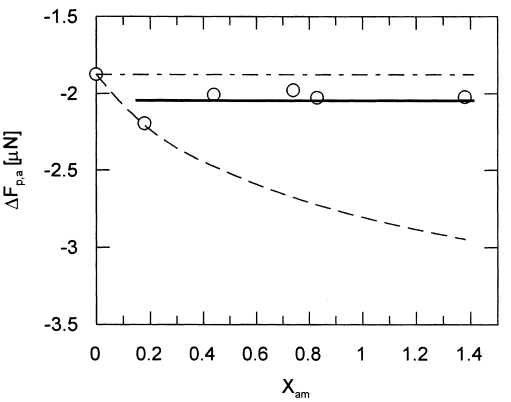


Fig. 11. Difference of forces ($F_{p,a}$ obtained using Eq. (3) for the gel phase at the reduced temperature $T_r = -0.01$ as a function of the anaesthetic:phospholipid molar ratio in the whole sample. For description and symbols see Fig. 10.

volume of heptacaine is caused by the presence of voids both in the fluid and gel bilayer of DPPC which are filled-in by the anaesthetic molecules at low anaesthetic concentrations. By taking the averaged values of $\Delta F_{p,a}$ for $X_{am} > 0$, one obtains that heptacaine is able to fill-in the voids which volume is $0.9 \pm 0.4\%$ and $0.5 \pm 0.3\%$ of the phospholipid volume in the liquid crystalline phase and in the gel phase of DPPC bilayers, respectively. This is substantially less than the volume of voids which hexane is able to fill-in [45]. This difference could be caused by several factors. Hexane is smaller, more flexible and more hydrophobic than heptacaine so that it can penetrate into defects in the bilayer hydrophobic region ($\sim 1$ nm wide zone at the centre of bilayer in case of hexane [45]) which are not accessible to heptacaine, and adapt conformationally to their geometry, while heptacaine is anchored at the lipid–water interface. Heptacaine could, thus, fill-in mainly lateral defects suggested to occur in bilayers by several authors and directly observed by small-angle neutron-scattering experiments (see [54,55] and references therein).

Anaesthetics affect not only the gel–liquid crystal phase transition, but also bilayer thickness [19,46], fluidity [17,47–49], and transitions between the bilayer and non-bilayer phases [49–52]. The consequence of the filling-in of the voids in lipid bilayer (if observed) for the lipid model of anaesthesia is obvious: Let us suppose that the anaesthesia in vivo is caused by the low concentration of anaesthetic molecules in the bilayer of target membrane-lower than corresponding to the volume of filled-in voids. Consequently, the physical mechanism responsible for the final effect cannot be a modulation of bilayer thickness or formation of non-bilayer structures (which occurs at high concentrations of anaesthetic in the bilayer). In this case the mechanism could be for example a modulation of the gel–liquid crystal phase transition temperature or/and the bilayer fluidity. Boulanger et al. [53] have calculated the tertiary amine local anaesthetic tetracaine (structurally similar to heptacaine) concentration in the lipid bilayer at concentrations used clinically. They have found that the corresponding anaesthetic:lipid molar ratio is $N_{af}:N_{pf} = 0.05 \div 0.4$ in the liquid crystalline phase. Our experiments show that the anaesthetic volume in the bilayer is close to that in the aqueous solution within this range. If the anaesthesia is in fact caused by such a high anaesthetic concentration in the bilayer, then our

results indicate that the modulation of bilayer thickness or formation of non-bilayer structures could play an important role in its mechanism.

## Acknowledgements

This study was supported by the grants of Slovak Scientific Grant Agency No. 2083 and No. 4173. The authors wish to thank J. Čižmárik (Comenius University, Bratislava) for the kind gift of anaesthetic, P. Balgavý (Comenius University, Bratislava) for stimulation of our investigation and for useful advises and M. Antalík (Institute of Experimental Physics, Košice) for the support of this work.

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