Influence of the Local Anesthetic Tetracaine on the Phase Behavior and the Thermodynamic Properties of Phospholipid Bilayers

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ABSTRACT We investigated the influence of the local anesthetic tetracaine on the thermodynamic properties and the temperature- and pressure-dependent phase behavior of the model biomembrane 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine by using volumetric measurements at temperatures ranging from 0° to 40°C and at pressures from ambient up to 1000 bar. The pVT measurements were complemented by temperature-dependent differential scanning calorimetric measurements. Information about the influence of different concentrations of the local anesthetic on the thermodynamic changes accompanying the lipid phase transitions, and on the thermal expansion coefficient, the isothermal compressibility, and the volume fluctuations of the lipids in their different phases, could be obtained from these experiments. The incorporation of tetracaine leads to an overall disordering of the membrane, as can be inferred from the depression of the main transition temperature and the reduction of the volume change at the main lipid phase transition. The expansion coefficient α_p and the isothermal compressibility χ_T of the lipid bilayer are enhanced by the addition of tetracaine and strongly enhanced values of α_p and χ_T, and the lipid volume fluctuations are found in the direct neighborhood of the main phase transition region. As tetracaine can be viewed as a model system for amphiphilic molecules, these results also provide insight into the general understanding of the physicochemical action of amphiphilic molecules on membranes. The experimental results are compared with recent theoretical predictions for the phase behavior of anesthetic-lipid systems, and the biological relevance of this study is discussed.

INTRODUCTION

The molecular mechanism of the anesthetic action on nerve membranes is still poorly understood. It is still controversial whether the membrane proteins or the surrounding lipid matrix are the primary target sites of anesthetic action (Roth, 1979; Ueda and Kamaya, 1984; Franks and Lieb, 1987). An important key in understanding the molecular mechanism of anesthesia might be the antagonistic effect of hyperbaric pressures against anesthetic action, which has been observed in vivo (see, e.g., Johnson and Flagler (1951) and Lever et al. (1971)). The discovery, that anesthesia can be reversed by application of hydrostatic pressure in vivo, initiated several studies of the pressure effect on the structural properties of anesthetic-lipid systems, recently (Auger et al., 1987, 1988a, 1988b, 1990; Winter et al., 1991; Böttner et al., 1992; Driscoll et al., 1991; Peng and Jonas, 1992). To elucidate the effect of local anesthetics on the thermodynamic properties of the pure lipid matrix only, we performed calorimetric and volumetric measurements on the model membrane 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)[1] containing the local anesthetic tetracaine (TTC). The experiments on TTC-DMPC dispersions, which are presented here, were performed at pH 9.5, where the anesthetic is in its uncharged form. By ^{2}H NMR studies it has been directly shown (Boulanger et al., 1981; Auger et al., 1988a) that the anesthetic intercalates partially into the bilayer at that pH value (see Fig. 1), and estimates of the molecular order parameter indicate that it is located with its long axis approximately parallel to the director of the lipid acyl chains. The partition coefficient, which is the ratio of the anesthetic concentration in the lipid to that in the aqueous phase, is about 600 for uncharged tetracaine in phospholipid bilayers, which means that more than 99% of the TTC is incorporated in the lipid bilayer.

Phospholipids in excess water exhibit a variety of thermotropic phase transitions, such as the temperature-dependent lamellar gel to gel ($L_{\beta'}$-$P_{\beta'}$) pretransition and the gel to liquid-crystalline ($P_{\beta'}$-L_α) main transition at higher temperatures (Cevc and Marsh, 1987). In the liquid-crystalline state, the hydrocarbon chains of the lipid bilayers are conformationally disordered, but the average chain orientation is perpendicular to the bilayer surface. In the gel phases, the hydrocarbon chains are extended and relatively ordered. However, the lipid molecules can differ in bilayer surface structure and lipid chain packing. The $P_{\beta'}$-gel phase, which exists between 14° and 24°C for DMPC, has a two-dimensional lattice structure in which the lipid bilayers are distorted by a periodic ripple in the plane of the lamellae, whereas the lower temperature $L_{\beta'}$ gel phase exhibits a planar bilayer surface. In addition to these thermotropic phase transitions, further pressure-induced phases have been observed (for a review, see, e.g., Wong et al. (1988), Braganza and Worcester (1986), Winter et al. (1989a, 1989b), Driscoll et al. (1991)). These phospholipid membrane phase transitions, and how they are affected by the incorporation of other species interacting with these membranes, have attracted considerable experimental attention, because they intimately reflect the molecular interactions of the membrane and may thus help in understanding membrane systems and function on a molecular level.

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Abbreviations used: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; TTC, tetracaine; DSC, differential scanning calorimetry.

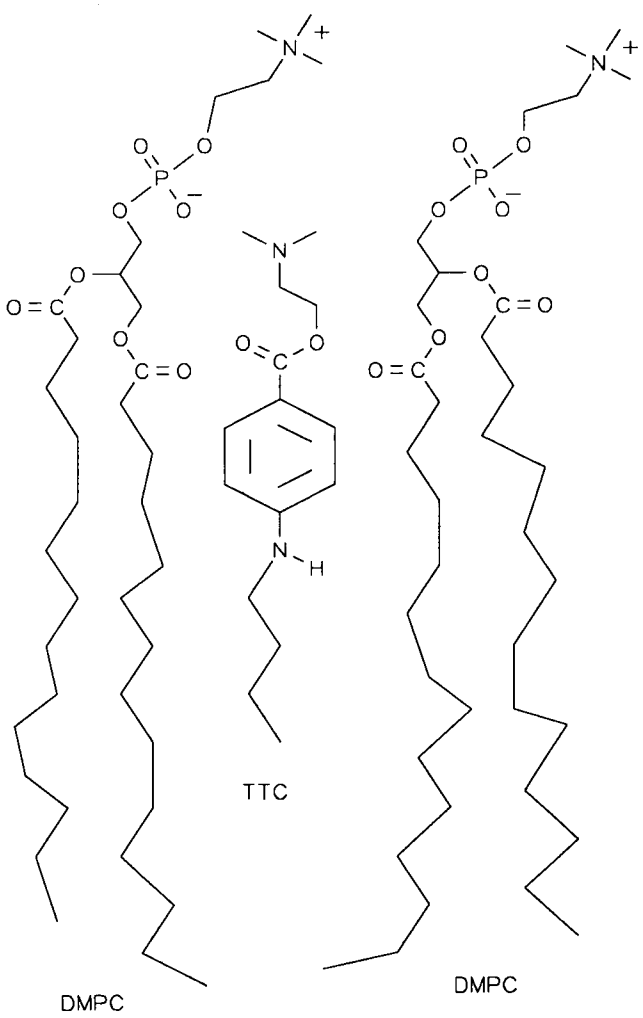

FIGURE 1 Schematic drawing of the position of the local anesthetic TTC in DMPC at pH 9.5 (adopted from Boulanger et al. (1981)).

For a complete understanding of the phase behavior of these lipid systems, temperature-dependent calorimetric and volumetric data are required at different pressures. However, only a few studies have been performed so far and these were mainly restricted to low pressures and to pure lipid systems, only (Nagle, 1973; Srinivasan et al., 1974; Liu and Kay, 1977; MacDonald, 1978; Nagle and Scott, 1978; Nagle and Wilkinson, 1978; Melchior et al., 1980; Russel and Collings, 1982; Schmidt and Knoll, 1985; Vennemann et al., 1986; Tosh and Collings, 1986; Wiener et al., 1988; Nagle and Wiener, 1988; Raudino et al., 1990; La Rosa and Grasso, 1990; Scarlata, 1991; Utoh and Takemura, 1985). Also recent theoretical work has elucidated the strong need for experimental volumetric data of lipid bilayer systems (Mouritsen, 1991).

In this paper, we report on volumetric measurements in the temperature range of 0–40°C at pressures from 1 to 1000 bar and on temperature-dependent calorimetric measurements which yield information about the influence of the local anesthetic tetracaine on the thermodynamic properties and the phase behavior of the model biomembrane DMPC as a function of anesthetic concentration over a wide range of temperatures and pressures.

EXPERIMENTAL PROCEDURES

Materials

DMPC and tetracaine hydrochloride were purchased from Avanti Polar Lipids (Birmingham, AL) and Sigma Chemical Co. (St. Louis, MO), respectively, and were used without further purification.

Sample Preparation

For pure phospholipid samples, DMPC was dispersed in distilled water above the gel to liquid-crystalline phase transition temperature of 24°C (Cevc and Marsh, 1987), and a homogeneous milky dispersion was formed by extensive vortexing. For the preparation of the sample with tetracaine, a sodium borate-phosphate-citrate buffer was used. The pH of the solution was adjusted to 9.5 with concentrated NaOH. The DMPC was dispersed in the buffer and then TTC was added. The resulting samples were subjected to five freeze-thaw-vortex cycles to ensure complete equilibration of TTC between the bilayer and aqueous phase. The sample pH was measured afterwards and readjusted if necessary.

Calorimetric Measurements

The calorimetric measurements were carried out with a Perkin-Elmer DSC 7 differential scanning calorimeter (DSC). About 20 mg of the lipid mixture were hydrated with 80 μl of buffer, containing the desired concentration of TTC, and then filled into DSC capsules, made from stainless steel with O-ring sealing. The samples were heated together with a blank at a programmed constant heating rate of 2°C/min. In the thermograms the difference between the heat flow into the sample and blank is plotted versus increasing temperature, which is proportional to the specific heat c_p of the sample. In order to evaluate the phase transition temperatures, a straight line was fitted to the upward deflection of the transition curves. The enthalpies of transitions were evaluated from the area under the calorimetric curves.

Volumetric measurements

The pVT measurements were carried out with a home-built high pressure cell (Böttner et al., 1993). The cell is made from stainless steel and has been designed for measurements in the temperature range from 0° to 100°C and at pressures from 1 to 2500 bar. A 5-wt% mixture of lipid in distilled water is prepared, degassed, and filled into the cell holding about 7 cm^3. A bellow made from stainless steel separates the sample volume from the pressurizing medium. The volume change of the lipid dispersion is measured as a function of pressure and temperature by the elongation of the bellow, employing an inductive method. The pressure is applied by means of a screw-type pressure generator and recorded by a Heise pressure-gauge. Temperature control is achieved by circulating water from a thermostat through the outside jacket of the pressure cell. The temperature is controlled within $\pm$ 0.1°C, and the pressure is accurate to $\pm$10 bar. In order to obtain absolute volumetric data, calibration measurements with a sample of well known volumetric data have to be performed. We used water, because its pVT data are known in a wide range of pressures and temperatures with high precision (Cho et al., 1991). The apparent specific volume V_L of the dispersed lipid is obtained by substracting the corresponding water value, knowing the density of water and the mass fraction of the lipid in the dispersion. It normally is more appropriate to report partial specific volumes when dealing with solutions. However, for bilayer dispersions in excess water there seems to be no essential difference between partial and apparent specific volumes (Wiener et al., 1988).

RESULTS AND DISCUSSION

Fig. 2 exhibits a selection of calorimetric scans of DMPC dispersions as a function of tetracaine concentration, given in mol% TTC with respect to lipid. Clearly, the well known main transition, corresponding to the $P_{\beta'}$ gel to liquid-crystalline bilayer conversion, of pure DMPC liposomes is seen at $T = 24°C$, and the smaller pretransitional endotherm peak, which is due to the $L_{\beta'}$ gel to $P_{\beta'}$ gel phase transition, occurs at about 14.5°C. The pretransition is accompanied with a small enthalpy change of about 3 kJ/mol, whereas the enthalpy change at the main transition comes to $\Delta H_m \approx 24$ kJ/mol. These data for the pure lipid system are in good agreement with data reported in the literature (Cevc and Marsh, 1987). The pretransition is already abolished by addition of about 3 mol% TTC. The main transition temperature T_m becomes continuously depressed with the addition of up to 10 mol% TTC, with little modification of the enthalpy change for the main transition, however. Parallel to the depression of T_m, the half width of the DSC signal broadens, indicating that the cooperativity of the transition is reduced. The cooperativity unit size (Sturtevant, 1987) of the lipid molecules is reduced by about 60% upon addition of, e.g., 10 mol% TTC. Obviously, chain disorder increases upon addition of TTC by increasing the number of gauche conformers in the lipid hydrocarbon chains. A similar conclusion has been drawn from ^2H NMR experimets on TTC/DMPC dispersions (Boulanger et al., 1981). At higher anesthetic concentrations, rather complex DSC peak structures appear, which indicate a demixing process of the lipid system. At concentrations above about 50 mol% TTC, different lipid supramolecular structures appear, such as mixed micelles from lipid and TTC, as can be inferred from small-angle neutron diffraction experiments (Winter et al., 1991; Böttner et al., 1992).

Fig. 3 exhibits the temperature dependence of the apparent specific lipid volume V_L of a DMPC dispersion containing 3 mol% TTC in comparison to V_L of the pure lipid system. With increasing temperature, the lipid volume increases. The change of V_L of pure DMPC near 14°C corresponds to a small volume change in course of the $L_{\beta'}$-$P_{\beta'}$ gel to gel transition (see also: Nagle and Wilkinson (1978), Schmidt and Knoll (1985), Vennemann et al. (1986)). Clearly, the gel to liquid-crystalline phase transition at 24°C can be seen, which

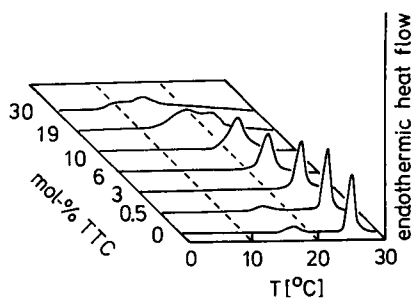

FIGURE 2 DSC scans of different TTC-DPMC-H_2O mixtures at pH 9.5 (scan rate, 2°C/min).

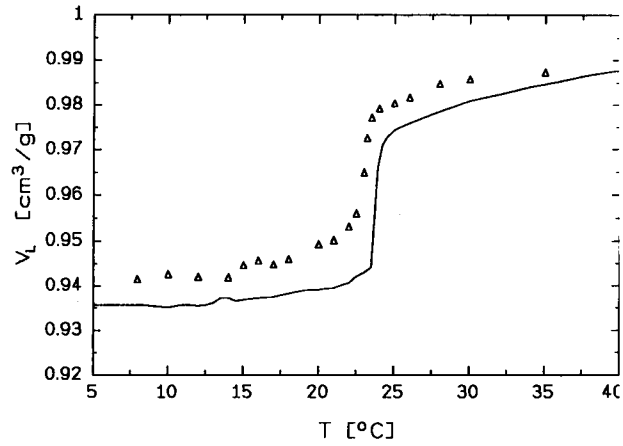

FIGURE 3 Temperature dependence of the apparent specific lipid volume V_L of a pure DMPC (*solid line*) and 3 mol% TTC/DMPC (△) dispersion at 1 bar and pH 9.5.

is accompanied by a 3% volume increase ($\Delta V_m = 0.028 \pm 0.001$ cm³/g). This volume change in course of the main transition is mainly due to changes of the chain cross-sectional area, because chain disorder drastically increases at the transition point. Assuming that the lipid volume can be partitioned into two parts, the volume of the lipid headgroup region and that of the hydrocarbon chains, and by noting that the volume of the headgroup is independent of temperature in the temperature region covered (Tardieu et al., 1973), a mean volume increase of 1.5 Å³ can be calculated for the methylen groups at the main transition. Beyond the main transition, a continous increase in lipid volume in the disordered liquid-crystalline phase is observed and the $V_L(T)$ curves seem to level off at higher temperatures.

It is clearly visible that the main transition is shifted toward a lower temperature by the addition of the local anesthetic. The volume change at the main transition is about 15% smaller than that of the pure lipid system. Fig. 4 displays the

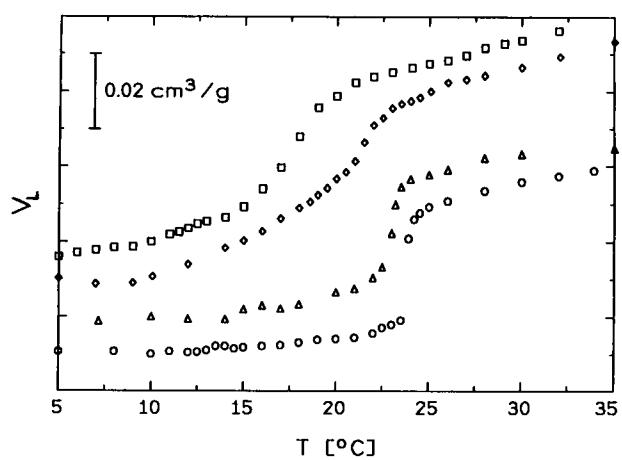

FIGURE 4 Temperature dependence of the apparent specific lipid volume V_L of pure DMPC (○), 3 mol% TTC/DMPC (△), 10 mol% TTC/DMPC (◇), and 20 mol% TTC/DMPC (□) dispersions at 1 bar and pH 9.5 (the curves are arbitrarily offset).

corresponding $V_L(T)$ curves of the membrane containing still higher concentrations of TTC. It can easily be seen that the main transition is shifted further toward lower temperatures and the transition width increases with increasing concentration of TTC. These results match the trends observed in the temperature-dependent calorimetric measurements, where, parallel to the depression of the main phase transition temperature T_m, the peak width of the DSC signal broadens with increasing anesthetic concentration.

Fig. 5 exhibits the isobaric thermal expansion coefficient α_p of the lipid system over the whole temperature range measured, which has been obtained from the data shown in Fig. 3. Contrary to the expansion coefficient of the liquid-crystalline phase below 30°C ($\alpha_p = 1.2\ (\pm 0.2) \times 10^{-3}$/°C) and of the $P_{\beta'}$ gel phase, α_p is much less in the $L_{\beta'}$ gel phase ($\alpha_p \approx 7 \times 10^{-4}$/°C). At the main transition, α_p increases up to a value of almost 6×10^{-2}/°C.

The expansion coefficient α_p of the 3 mol% TTC-containing sample, which is also displayed in Fig. 5, drastically increases relative to that of the pure lipid system in the gel phase. As can be seen from Fig. 6, with increasing anesthetic concentration, strongly enhanced values of α_p are found below and above the transition point, whereas its maximum value at T_m is reduced.

As an example of a pressure-dependent volumetric study, Fig. 7 displays $V_L(p)$ of DMPC and of the 3 mol% tetracaine-containing sample at $T = 30$°C. By increasing the pressure, the phase transition of pure DMPC into the ordered gel-phase can be induced as can be seen from the abrupt decrease of the lipid volume within a rather narrow pressure range of about 10 bar around $P = 265$ bar. The accompanying volume change is $\Delta V_m = 0.023$ cm³/g.

The incorporation of the anesthetic into the DMPC bilayer causes an about 15% decrease of ΔV_m relative to that of the pure lipid system; it broadens the transition about 4-fold and shifts the pressure-induced liquid-crystalline to gel phase transition toward higher pressures. The slope dT/dp of the main transition curve of the 3 mol% TTC-containing sample is similar to that of the pure lipid system: $dT/dp = 21 \pm 0.5$°C/kbar.

Whereas the temperature-induced main transition of DMPC containing 10 and 19 mol% TTC is clearly observable in the DSC tracing of Fig. 1, the pressure-induced main transition in these samples cannot be detected in the plots of V_L vs. p (see Fig. 8), as the transitions extend over a too wide pressure range.

From the volumetric data of Fig. 7, the isothermal compressibility coefficient χ_T of the lipid systems can be calculated (Fig. 9). It appears that the compressibility of the $P_{\beta'}$ gel phase of pure DMPC is substantially lower than that of the L_α phase (at $T = 30$°C: $\chi_T(P_{\beta'}) = 5\ (\pm 2) \times 10^{-5}$/bar and $\chi_T(L_\alpha) = 14\ (\pm 2) \times 10^{-5}$/bar). As can be also seen from Fig. 9, χ_T drastically increases approaching the main transition point, does not show a sharp discontinuity, however. Actually, a sharp discontinuity has never been observed in any thermodynamic or dynamic property at the phospholipid

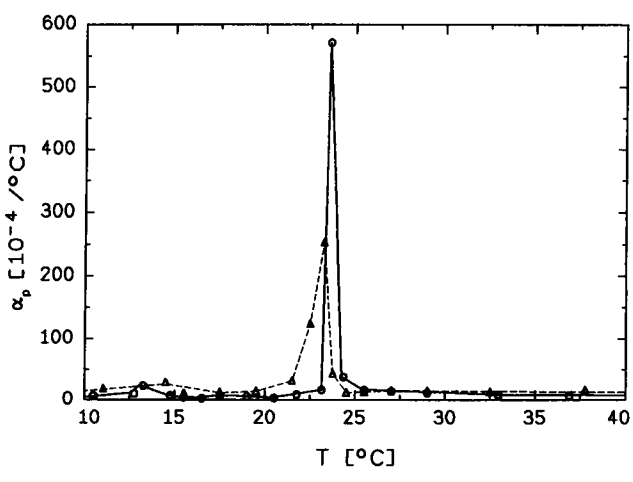

FIGURE 5 Thermal expansion coefficient α_p of pure DMPC (●) and 3 mol% TTC/DMPC (△) in H_2O as a function of temperature ($p = 1$ bar).

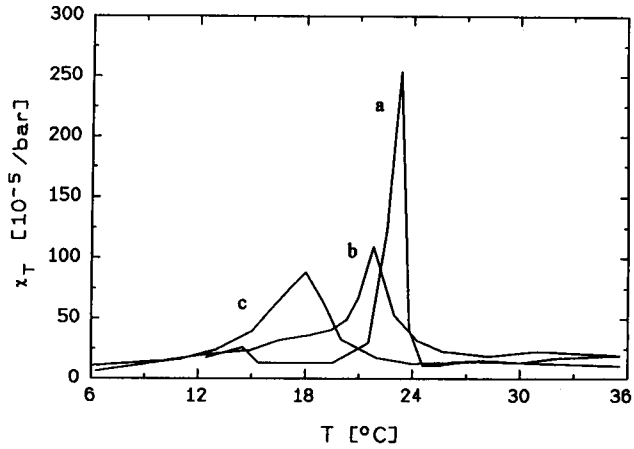

FIGURE 6 The thermal expansion coefficient α_P of (*a*) 3 mol% TTC/DMPC, (*b*) 10 mol% TTC/DMPC, and (*c*) 20 mol% TTC/DMPC dispersions at $p = 1$ bar and pH 9.5.

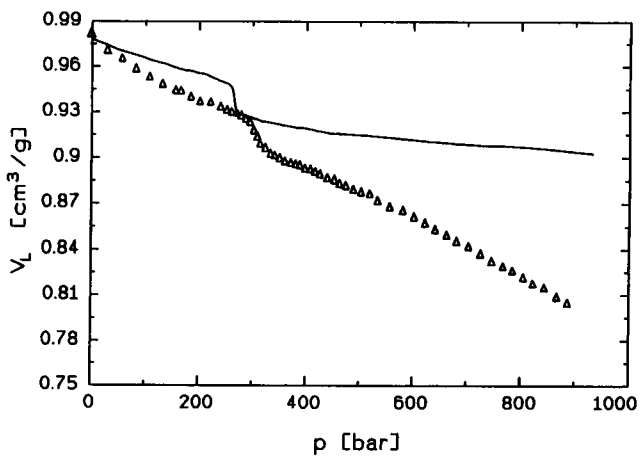

FIGURE 7 Comparison of the apparent specific volumes V_L of a DMPC (*solid line*) and a 3 mol% TTC/DMPC (△) multilamellar dispersion as a function of pressure at $T = 30$°C and pH 9.5.

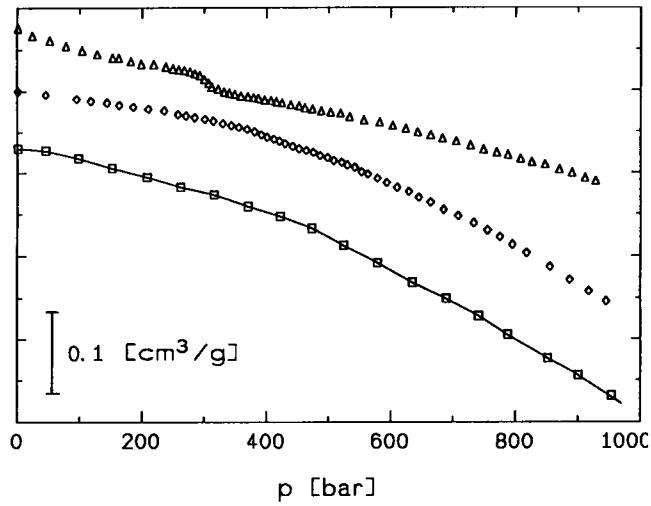

FIGURE 8 Change of specific lipid volume V_L of a (*a*) 3 mol% TTC/DMPC (△), (*b*) 10 mol% TTC/DMPC (◇), and (*c*) 20 mol% TTC/DMPC (□) dispersion as a function of pressure at $T = 30°C$ and pH 9.5 (the curves are arbitrarily offset).

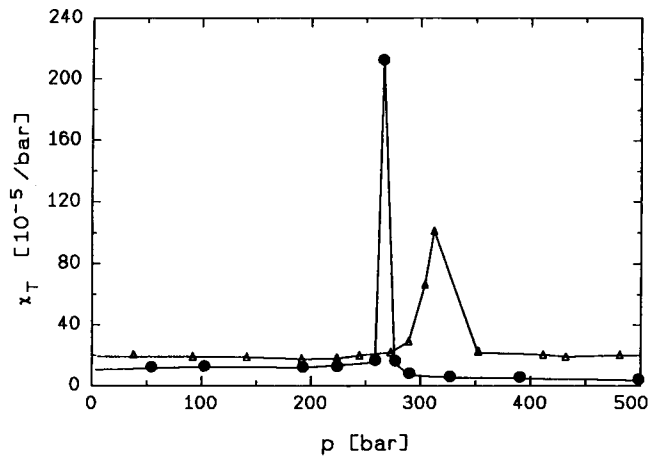

FIGURE 9 Isothermal compressibility χ_T of a pure DMPC (●) and 3 mol% TTC/DMPC (△) dispersion at, e.g., $T = 30°C$ and pH 9.5.

main phase transitions. This has led to the proposal that the main transition might be pseudocritical, i.e., in principle of first order but very close to a critical end point and consequently influenced by thermal density fluctuations. The importance of fluctuations near T_m has been described theoretically, employing a multistate Pink-Potts model (Mouritsen, 1991; Ipsen et al., 1990a, 1990b).

Larger values for the compressibilities are found for both lipid phases by addition of 3 mol% TTC, and there is no apparent difference in the coefficient of compressibility between the gel and liquid-crystalline phases for DMPC plus tetracaine. χ_T is drastically reduced at the main transition point, however enhanced in the direct neighborhood of the transition, similar to the behavior found for the expansion coefficients of the TTC-containing samples in the transition region. As can be inferred from the $V_L(p)$ curves in Fig. 8, the compressibilities of the samples containing 10 and 19 mol% TTC are larger in the gel phase in comparison to that of the sample with 3 mol% TTC.

A further important thermodynamic quantity that can be obtained from the isothermal compressibility data is the mean square volume fluctuation of the lipid in its different phases, which is given by $\langle \delta V_L^2 \rangle = k_B T V_L \chi_T$, where T is the absolute temperature and k_B the Boltzmann constant (Landau and Lifschitz, 1987). The relative root mean square fluctuation of the lipid volume $\delta V_{L,rel} = (\langle \delta V_L^2 \rangle)^{1/2}/V_L$ is displayed in Fig. 10 as a function of pressure. $\delta V_{L,rel}$ in the liquid-crystalline phase of pure DMPC is about 7%; it increases up to about 30% at the main transition and decreases to 4% in the gel phase. In comparison to volume fluctuations of other biochemical systems, like proteins (Gekko and Hasegawa, 1986a, 1986b), these relative volume fluctuations of the lipids in membranes are large. They are smaller, however, in comparison to those of pure water. This fact may be of significant biological relevance for the understanding of the dynamics, structure, and function of membrane bound proteins. The addition of 3 mol% of the local anesthetic leads to an increase of the relative lipid volume fluctuations up to about 9% in both lipid phases. At the transition point, $\delta V_{L,rel}$ is diminished in comparison to the value of the pure lipid system, however.

In a theoretical modelling (Mouritsen, 1991), it could be shown that the binding of foreign molecules, like anesthetics, in bilayer membranes might strongly couple to the thermal density and concentration fluctuations of the lipid system near its gel to liquid-crystalline phase transition, thus leading to a strong enhancement of $\delta V_{L,rel}$ in the neighborhood of T_m, which has in fact been observed experimentally. These findings are also of important biochemical relevance, as in lipid bilayer membranes, strong density or concentration fluctuations are related to the transmembrane permeability of ions and small molecules.

Although the biochemical action of local anesthetics is still controversial as to whether or not the action is lipid mediated, one thing which is clear, however, is that local anesthetics do

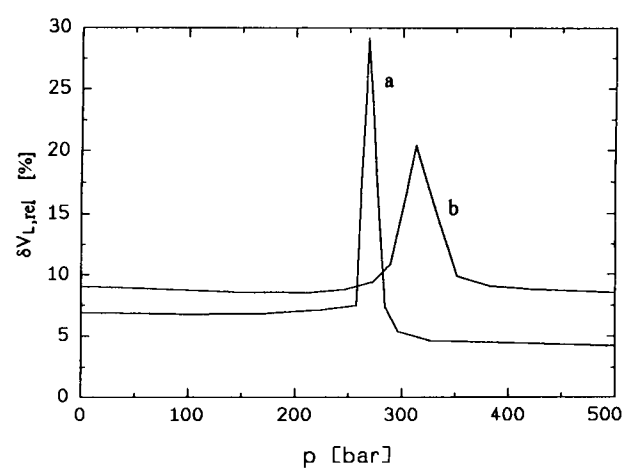

FIGURE 10 Relative mean volume fluctuations $\delta V_{L,rel}$ of (*a*) DMPC and (*b*) 3 mol% TTC/DMPC as a function of pressure at $T = 30°C$ and pH 9.5.

strongly perturb the lipid bilayer system and change their phase behavior, their thermodynamic and thermomechanic properties, to an extent which often can be correlated with the potency of the agent (Ueda et al., 1977).

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