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# Calorimetric studies of the thermodynamic properties of lamellar lecithin/local anaesthetic/water mixtures

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# Calorimetric studies of the thermodynamic properties of lamellar lecithin/local anaesthetic/water mixtures

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Differential scanning calorimetry has been used to study the effects of two different local anaesthetics from heptacaine type (abbreviated HK and IR-1) on the thermodynamic properties of dipalmitoyl lecithin in bilayers. The model membrane systems studied were multilamellar aqueous dispersions of unbranched and branched dipalmitoyl lecithin and their mixtures in the presence of the local anaesthetics. The calorimetric results show that both anaesthetics differ qualitatively in their miscibility effects. Incorporation of the anaesthetic HK into the bilayer from aqueous dipalmitoyl lecithin dispersions causes a large decrease of the main transition temperature (transition gel phase → $L_\alpha$ phase). For the anaesthetic IR-1, this decrease in the main transition temperature is considerably smaller. In accord with the phase diagram of the ternary system dipalmitoyl lecithin/heptacaine/water, demixing takes place in the low temperature phase at a mixing ratio $n_t$ of about 16/1 molecules of lecithin per molecule of heptacaine HK. However, if the anaesthetic IR-1 is used instead of HK, demixing in the gel phase does not occur until the mixing ratio has reached the value 1/1 molecule of lecithin per molecule of IR-1. Furthermore heptacaine effects the miscibility behaviour of the ternary system unbranched lecithin/branched lecithin/water. The miscibility gap within the gel phase is extended but the type of phase diagram is not changed.



## 1. Introduction

The interaction between phospholipid bilayers and anaesthetics represents a comprehensive field of biomembrane and model membrane research [1–23], but is still poorly understood. For instance local anaesthetics are known to exert their action by blocking the sodium channels of nerve membranes. However, whether this blocking is the result of a direct anaesthetic–protein interaction or a perturbation by the anaesthetics of the liquid-crystalline lipid matrix surrounding the channels is still unclear [24, 25].

Many techniques have been used to study the interactions of local anaesthetics with biomembranes and liquid-crystalline model membranes, including high resolution nuclear magnetic resonance [26], electron spin resonance [27–29], and neutron diffraction [30]. All of these studies suggest that the anaesthetic intercalates partially into the lipid bilayer. On the other hand, information about the effects of the local anaesthetic tetracaine on the order and dynamics of the lipid acyl chains has been obtained by $^2H$ NMR spectroscopy [31, 32]. These investigations indicate that the anaesthetic reduces the order parameter of the entire lipid acyl chains in the liquid-crystalline $L_\alpha$ phase. This disordering effect is larger in the presence of cholesterol and for the charged form of the tetracaine.

Nevertheless, little is known about the effects of local anaesthetics on the thermodynamic and structural properties of phospholipid bilayers in the gel state. Auger

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*et al.* [33] have discussed the effects of tetracaine on the structural and dynamic properties of lipids in multilamellar aqueous dispersions of phospholipids and phospholipid/cholesterol mixtures. The infrared spectra indicate that the effects of tetracaine on the structure of pure dimyristoyl lecithin bilayers in the gel state are dependent on the charge of the anaesthetic. The uncharged tetracaine disorders the lipid acyl chains while the charged form induces the formation of an interdigitated gel phase.

These results from literature were the starting-point of our investigations on the effects of two different local anaesthetics from heptacaine type on the miscibility behaviour of aqueous lecithin dispersions. We have used both unbranched and branched lecithins. The local anaesthetics differed in their chemical structures. We were interested in thermodynamic parameters of the phase transitions, the phase diagrams, and also in the influence exerted by the added anaesthetic on the hydration properties of the lecithin head group in the bilayers and in the miscibility properties of the ternary and quaternary systems. The thermodynamic investigations were performed in the concentration range saturated with water, e.g. in the so-called heterogeneous two phase region [34]. The local anaesthetics, chosen for our experiments, were the hydrocholoride of the 2-piperidino ethyl ester of carbanilic acid (IR-1) and heptacaine (HK), which represent the hydrocholoride of the 2-piperidino ethyl ester of basic 2-heptyloxycarbanilic acid. The homologous IR-1 proved to be a local anaesthetic with a relatively small efficiency. On the other hand the homologous HK is the most effective local anaesthetic [35].



## 2. Methods and materials

### 2.1. *Calorimetric measurements*

For the calorimetric measurements to determine the phase transition parameters, the phase diagrams and the hydration numbers of the lecithin/anaesthetic/water mixtures, a DSC-2 (Perkin–Elmer) was used. The methods as well as experimental and methodical details described in [36].

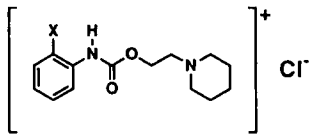
In the direction of increasing temperature between 240 K and 330 K, a peak called the ice peak appears within the DSC scans of the aqueous dipalmitoyl lecithin dispersions. By evaluating the dependence of its area on the mixing ratio lecithin/free water, the portions of free water and water, bound by the head groups, can be determined. The melting enthalpy ice → water being well-known, so the portion of water which participates in the melting process can be derived from the areas of the DSC curves. The difference between the amount of water in the lecithin/water dispersion is proportional to the water bound by the lecithin head groups. It allows us to calculate the molar ratio of bound water/lecithin and consequently the hydration numbers of molecules of water per molecules of lecithin.

### 2.2. *Sample preparation*

Dipalmitoyl lecithin and the anaesthetics were dissolved in a chloroform/methanol mixture (85 : 15 = v : v). The dissolved mixture was evaporated under vacuum by means of a rotary evaporator. Then the mixture was dried under vacuum condition ($\sim 1{\cdot}33 \times 10^{-2}$ Pa) at 350 K for 2 hours. 50 wt % distilled water was added to the dried samples, deposited in aluminium DSC pans and annealed for 2 hours above the main transition temperature of the dipalmitoyl lecithin/water mixture.

### 2.3. *Substances*

The following unbranched and branched lecithin 1,2-dipalmitoyl-DL-phosphatidylcholine (di-(C16:0)-PC) and 1,2-di-$C_1$-methyl-palmitoyl-DL-phosphatidylcholine, (di-(2$C_1$-C16:0)-PC) were used. The origin, synthesis, and purification of both lecithins have been described elsewhere [37]. Two homologues of local anaesthetics with the chemical formula




$X$ = H: hydrochloride of 2-piperidino ethyl ester of the basic carbanile acid, (IR-1) and $X$ = O–$C_7H_5$: hydrochloride of 2-piperidino ethyl ester of the basic 2-heptyloxy-carbanile acid, 'heptacain', (HK) were tested.

## 3. Results

### 3.1. *Phase diagram of the pseudo-binary system di-(C16:0)-PC/HK/$H_2O$*

In figure 1 selected DSC curves of singular mixtures with increasing heptacaine concentrations are shown; $n_r$ denotes the ratio of molecules of lecithin per molecule of anaesthetic. There are two different types of DSC curves. At low heptacaine concentration, only a single main transition peak is observed. When the heptacaine concentration is increased the peak splits into two or three peaks, a fact which indicates the demixing processes.

Within the concentration range tested in our experiments the system dipalmitoyl lecithin/water [34] undergoes a pretransition ($T_p$ = 309·5 K) and a main transition ($T_m$ = 315·8 K), the so-called gel to liquid-crystalline phase transition temperature. On adding only very small amounts of HK, i.e. molar ratios above $n_r$ 36/1, disappearance of pretransition peak is observed, accompanied by broadening of the main transition peak. From the positions of the main transition peaks it follows that the shift towards the lower final transition temperature increases with increasing HK content. The area ratios of peaks, caused by the splitting of the main transition peak, depend on the concentration ratios within the mixture. This is distinctly demonstrated at mixing ratios $n_r$ of 15/1, 9/1 and 7/1. Thus demixing phenomena in the low temperature phase occur at mixing ratios above $n_r$ of approximately 15/1.

The pseudo-binary phase diagram of di-(C16:0)-PC/HK/$H_2O$ in figure 2 shows the results from our DSC curves. The liquidus and the solidus curve in figure 2 show four regions at constant temperature. These constant temperatures are identical to those of the peak maxima. Up to a molar ratio $n_r$ of 16/1, the two components are completely miscible in both the high and the low temperature phase. The homogeneous phase is separated into clusters differing in their composition. Because of methodical restricted feasibilities for evaluating DSC curves the boundaries of these demixing regions cannot be determined precisely, but they may be estimated approximately by the following $n_r$ values of 16/1, 8/1, 3/1 and 2/1. At a mixing ratio $n_r$ of 5/1, for instance, the following equilibrium states will be bound toward increasing temperatures. In the region of the first horizontal line at 305·0 K clusters with compositions $n_r$ of 8/1 and 3/1, in the low temperature phase coexist with parts of the high temperature phase. As predicted by the lever rule, the portion of cluster with the composition $n_r$ of 3/1 is greater than that for $n_r$ of 8/1. On increasing the temperature

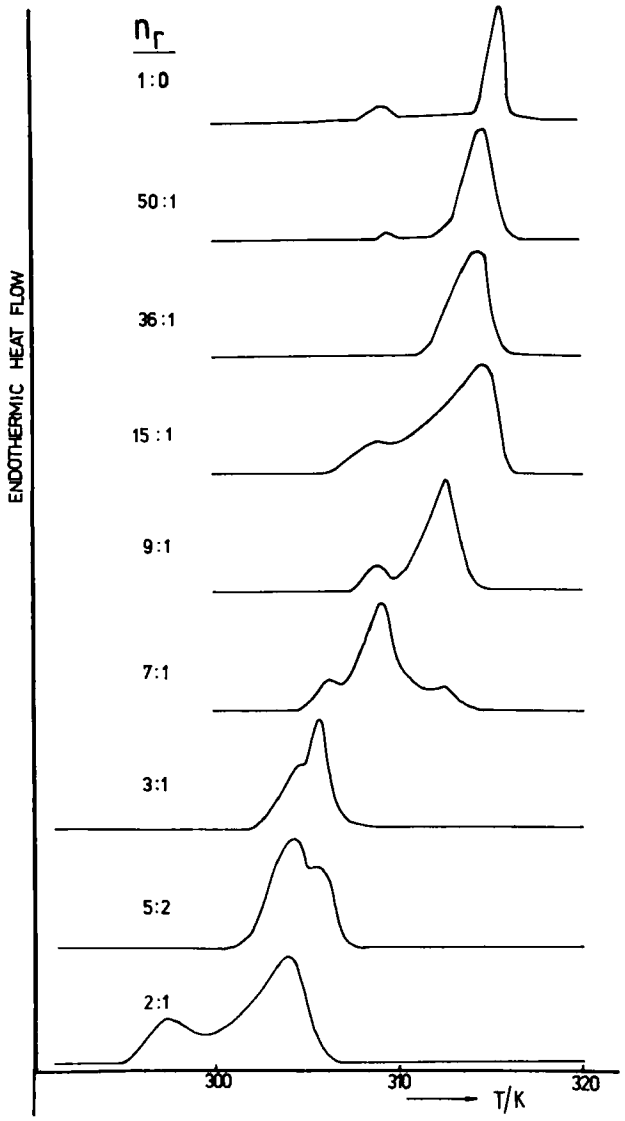



Figure 1. Selected DSC heating scans of mixtures of the ternary system dipalmitoyl lecithin/heptacaine/water (50 wt % water) showing the dependence on the heptacaine concentration. The ratio $n_r = n_{\text{di-(C16:0)-PC}}/n_{\text{HK}}$ means the quotient from moles of the dipalmitoyl lecithin and heptacaine. Heating rate: 1·25 K min$^{-1}$.

the clusters with the composition $n_r$ of 3/1 vanish and a second peak is observed within the DSC curves. On increasing the temperature this procedure goes on until the mixture is completely molten. The decrease in the final transition temperature on increasing the heptacaine concentration was also observed by other authors [13, 23].

Our results concerning the phase diagram are confirmed by increasing transition enthalpies $\Delta\bar{H}_U$ and hydration numbers of the mixtures. Figure 3 shows the dependence of $\Delta\bar{H}_U$ on the composition of the mixtures. As shown, $\Delta\bar{H}_U$ for the main transition differs only slightly within the region of complete miscibility. At mixing ratios above $n_r$ of approximately 16/1, the transition enthalpies increase remarkably.

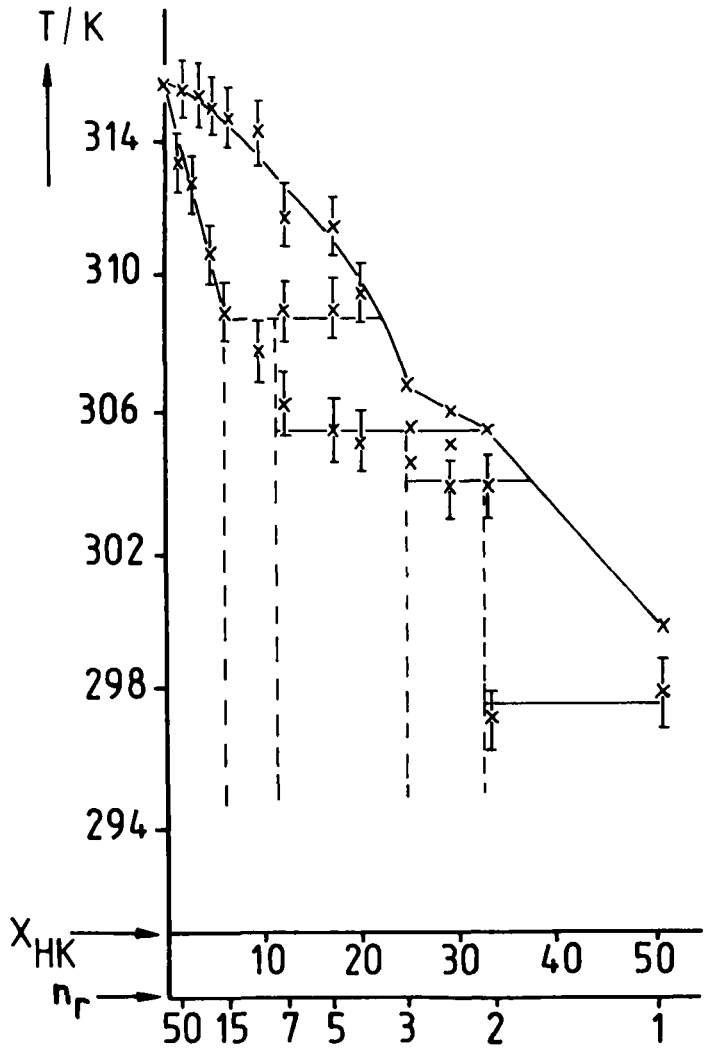



Figure 2. Phase diagram for the pseudo-binary system dipalmitoyl lecithin/heptacaine/water in the presence of excess water (50 wt %). The mole fraction $x_{HK}$ is related to the concentration of heptacaine (HK) by $n_r = n_{di(C16:0)\text{-}PC}/n_{HK}$.

This points to an elevation of positive deviations occurring simultaneously within this concentration range. This observation is in accord with the results already discussed in [36].

Alterations in the hydrated state of lecithin in the dependence on the concentration of added heptacaine will be of importance in connection with the phase diagram type and the molecular interpretation of the demixing phenomena. Figure 4 gives a survey of data resulting from evaluating the ice peak. The number of water molecules is plotted against the mole fraction of heptacaine. As already known from our measurements, di-(C16 : 0)-PC has a limit for hydration of about 10 molecules of water per molecule of lecithin [38]. On adding heptacaine the hydration numbers are increased to about 22 molecules of water per molecule of lecithin.

NMR measurements, performed by Abrahamsson *et al.* [39], also indicated an increase in the hydration numbers originating from added local anaesthetics. As

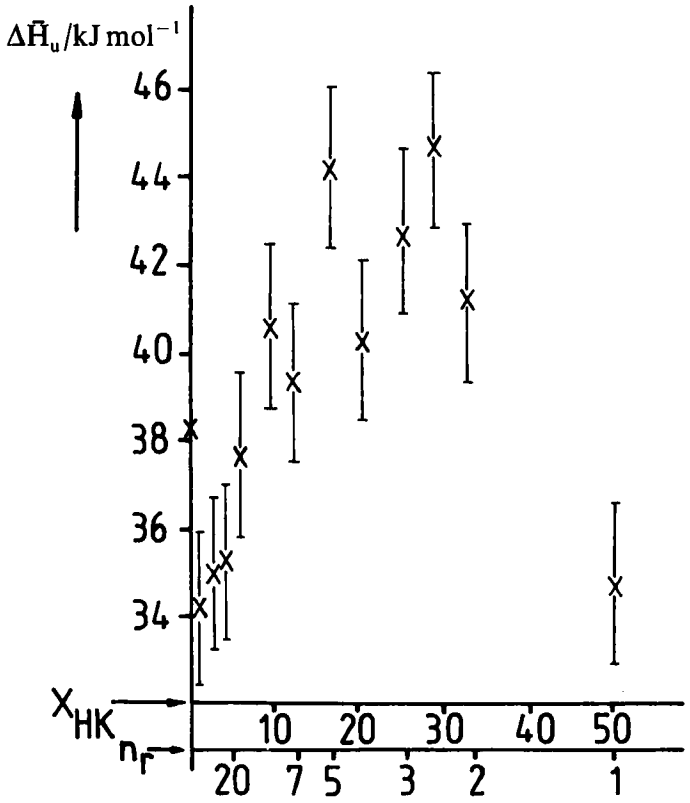



Figure 3. Concentration dependence of the transition enthalpies $\Delta\bar{H}_U$ (main transition) of mixtures in the ternary system dipalmitoyl lecithin/heptacaine/water in the presence of excess water (50 wt %). The mole fraction $x_{HK}$ is related to the heptacaine concentration by $n_r = n_{\text{di-(C16:0)-PC}}/n_{HK}$. The $\Delta\bar{H}_U$ values are related to the water free state.

proved by the deuterium quadrupolar splitting in egg–lecithin dispersions, added local anaesthetics caused an elevation of the maximum hydration number from 12·5 to 18. This might be caused by the ability of heptacaine to bind water molecules. On incorporation heptacaine into the bilayer, however, the positive charge of that molecule may repulse the positively charged part of the head groups in the lecithin molecules.

### 3.2. *Selected calorimetric measurements in the pseudo-binary system di-(C16 : 0)-PC/IR-1/$H_2O$*

Figure 5 shows selected DSC curves from singular mixtures. Increasing the IR-1 content systematically changes the curvature of the DSC scans. In contrast to the system discussed previously (compare figure 1), all of the mixtures now show a single main transition peak. The pretransition peak, characteristic of the system di-(C16 : 0)-PC/$H_2O$, without added IR-1 (transition $P_{\beta'} \rightarrow L_{\beta'}$ phase), is distinctly represented up to a molar ratio $n_r$ of 10/1. At mixing ratios above $n_r$ of 14/1, the pretransition temperature shifts towards higher values and the pretransition peak becomes a shoulder of the main transition peak. At mixing ratios above $n_r$ of 1/1, we observed a splitting of the main transition peak and a sudden reappearance of the pretransition.

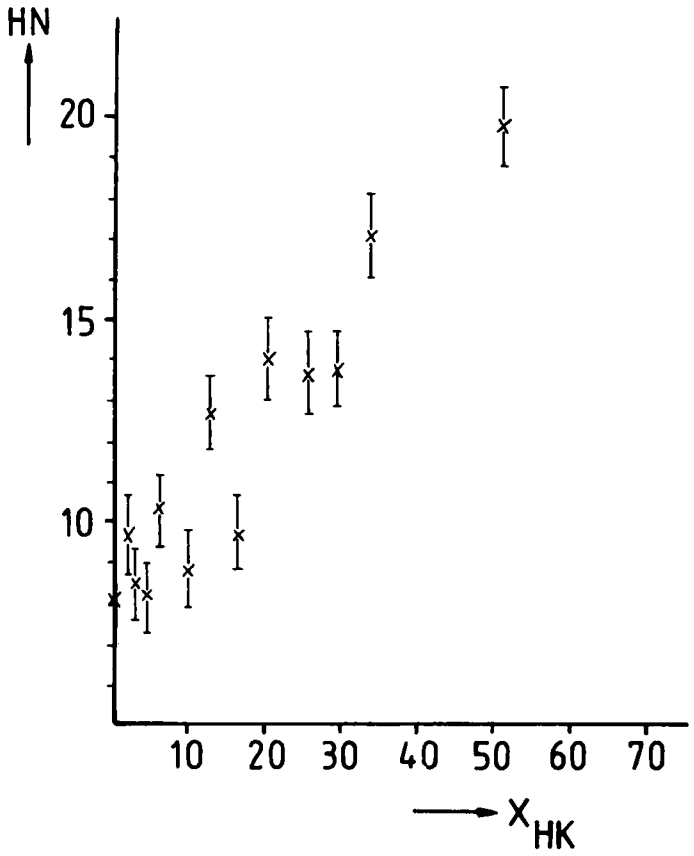



Figure 4. Graphical representation of the influence of the heptacaine-concentration $x_{HK}$ on the hydration number (molecules of water per molecule of dipalmitoyl lecithin). The mole fraction $x_{HK}$ is related to the heptacaine concentration.

Figure 6 shows curves, characteristic of the dependence of the maximum main transition temperature on the mixture composition, which may be of interest for the miscibility analysis. It is obvious that the maximum of the main transition temperature remains nearly constant up to a mixing ratio $n_r$ of 2/1, at higher concentrations it is shifted toward lower temperatures. The enthalpies of the main transition of the mixtures as shown in figure 7, are slightly elevated in comparison with those, resulting from the system lecithin/water without added IR-1 [34].

The hydration numbers, obtained by evaluating the ice peak, are illustrated graphically in figure 8. The elevation of the lecithin hydration number brought about by IR-1 is somewhat smaller in comparison with that of the heptacaine system. We found an increase from hydration number of 10 to 18. This suggests that IR-1 also enlarges the bilayer thereby making feasible additional water incorporation.

### 3.3. *Phase diagram of the pseudo-binary system di-(C16 : 0)-PC/di-(2$C_1$-C16 : 0)-PC/$H_2O$*

Resulting from our investigations, described in §§3.1 and 3.2, we checked the influence of the anaesthetic HK on the miscibility properties of the pseudo-binary system di-(C16 : 0)-PC/di(2$C_1$-C16 : 0)-PC/$H_2O$ at constant HK concentration. As a

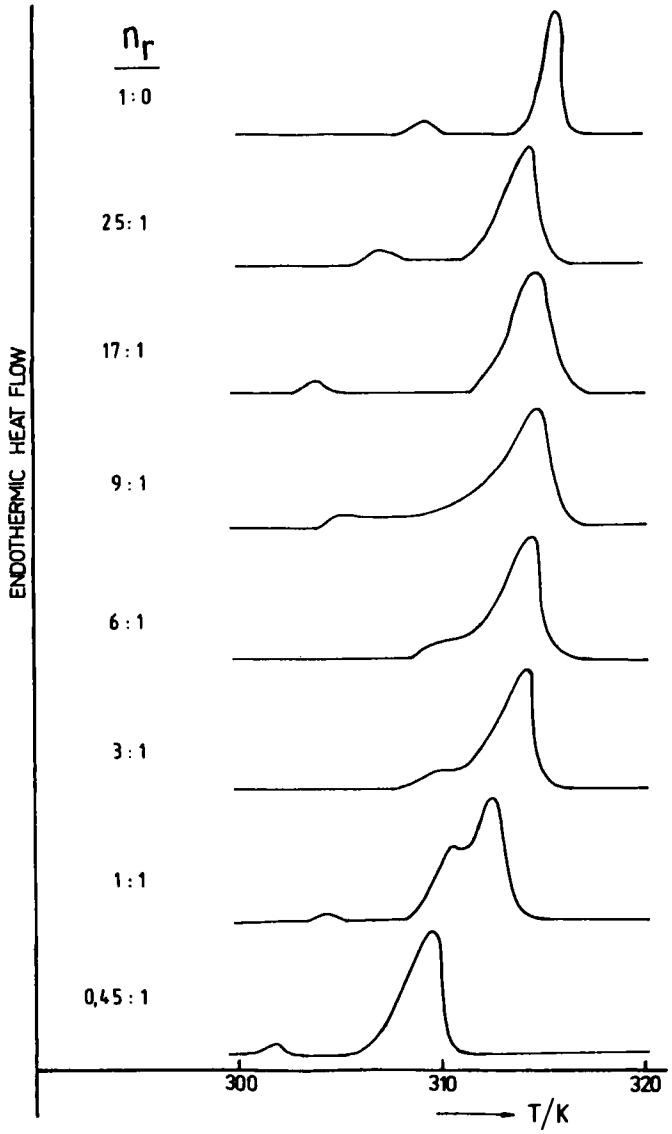



Figure 5. Selected DSC heating scans of mixtures in the ternary system dipalmitoyl lecithin/anaesthetic IR-1/water (50 wt % water) at a rate of 1·25 K $min^{-1}$ showing the dependence on the IR-1-concentration $n_r = n_{di(C16:0)\text{-}PC}/n_{IR\text{-}1}$.

first step we investigated the phase diagram of this ternary system without added HK. In figure 9 the phase diagram of the pseudo-binary system is shown. The solidus and liquidus curves demonstrate complete miscibility in the low temperature phase up to a mole fraction $x$ of 0·25. In the concentration range from $x$ of 0·25 to 0·55 we have observed a demixed low temperature phase, consisting of two separated fractions, a nearly equimolecular mixture of the two components and a fraction enriched with branched lecithins. With increasing mole fraction complete mixing occurs once more. In the high temperature, liquid-crystalline $L_\alpha$ phase, however, the two lecithins are completely miscible over the whole concentration range. The miscibility gap is situated in the concentration range of the component with the lower transition temperature and enthalpy. In addition the demixing state at the mole fraction of 0·6 was analysed

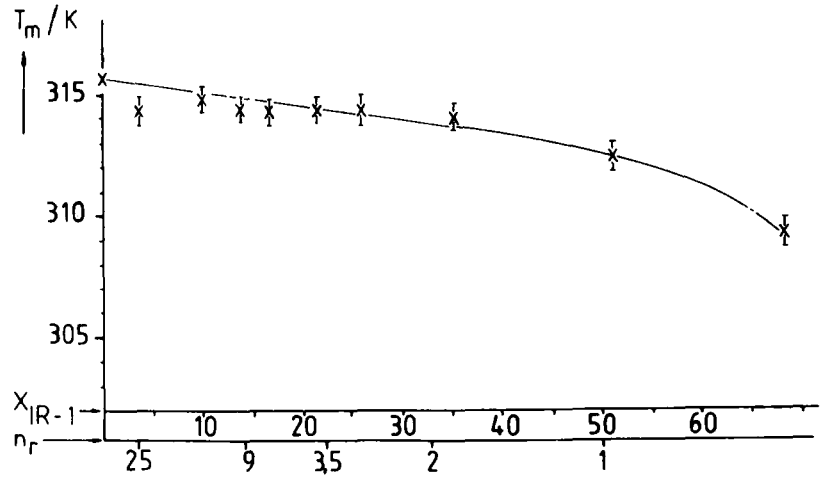



Figure 6. Graphical representation of the influence of the IR-1 concentration on the main transition temperature $T_m$. The mole fraction is related to the anaesthetic IR-1 concentration by $n_r = n_{di(C16:0)\text{-}PC}/n_{IR\text{-}1}$

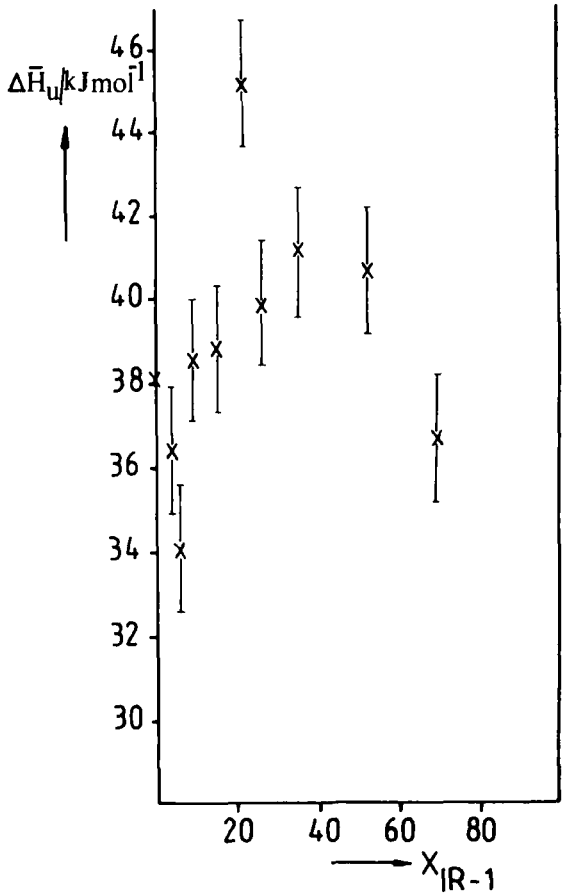



Figure 7. Concentration dependence of the transition enthalpies $\Delta\bar{H}_U$ (main transition) of mixtures in the ternary system dipalmitoyl lecithin/anaesthetic IR-1/water in presence of excess water (50 wt %). The mole fraction is related to the anaesthetic IR-1 concentration.

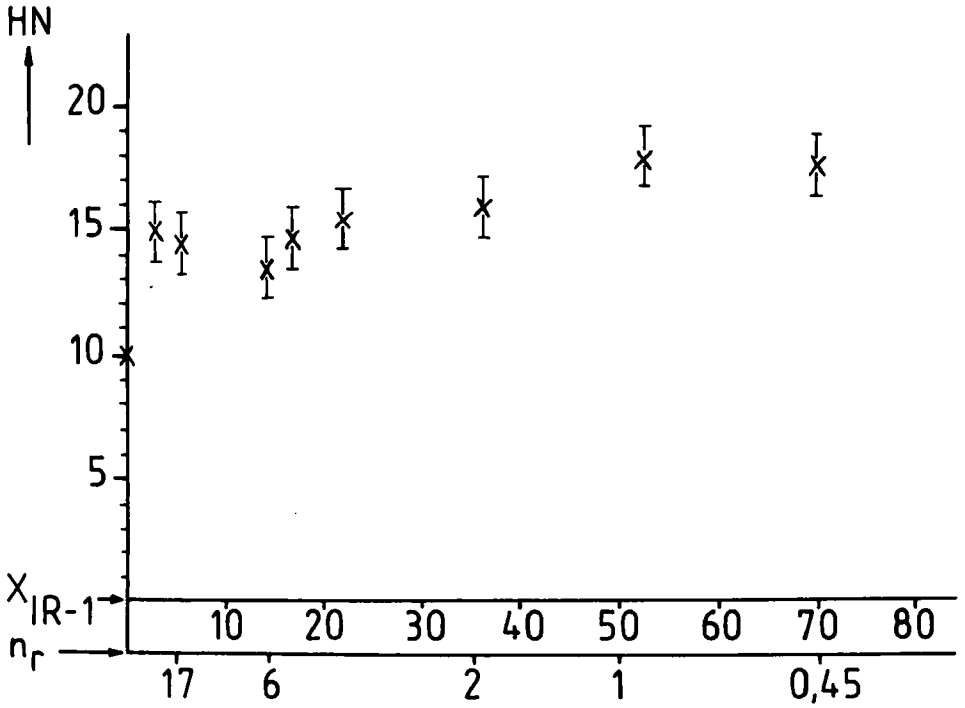



Figure 8. Graphical representation of the influence of the IR-1 concentration $x_{IR\text{-}1}$ on the hydration number (molecule of water per molecule of dipalmitoyl lecithin). The mole fraction is related to the anaesthetic IR-1 concentration by $n_r = n_{di\text{-}(C16:0)\text{-}PC}/n_{IR\text{-}1}$.

by the existence of a ripple structure in the electron micrographs, as described in [40, 41]. The presence of the ripple structure is an excellent criterion for miscibility or immiscibility properties of lecithins, which are able to form the $P_{\beta'}$-phase.

Another argument for the existence of the miscibility gap is the curvature in the $\Delta\bar{H}_U/x$ curve in figure 10. The experimental data cannot be described as an ideal mixture. As discussed in [36], the positive deviation from the straight line originates only in a positive amount of excess enthalpy $\Delta H_M^E$ of the mixtures. Therefore, such a shape of the $\Delta\bar{H}_U/x$ curve is also a suitable proof for the existence of miscibility gaps in the low temperature phase.

### 3.4. *Phase diagram of the quaternary system di-(C16 : 0)-PC/di-(2C$_1$-C16 : 0)-PC/HK/H$_2$O at constant H$_2$O and HK concentrations*

As described in §3.1, the calorimetric investigations on the system di-(C16 : 0)-PC/HK/$H_2O$ demonstrated that no pretransition occurred at a mixing ratio $n_r$ of 36/1. This is a suitable condition for our calorimetric investigation. For that reason we selected this mixing ratio in our first tests on the influence of heptacaine on the miscibilty behaviour of the binary system di-(C16 : 0)-PC/di-(2$C_1$-C16 : 0)-PC within the water-saturated concentration range (50 wt %) at constant HK concentration ($n_r$ = 36/1). The pseudo-binary phase diagram in figure 11 results from our calorimetric measurements.

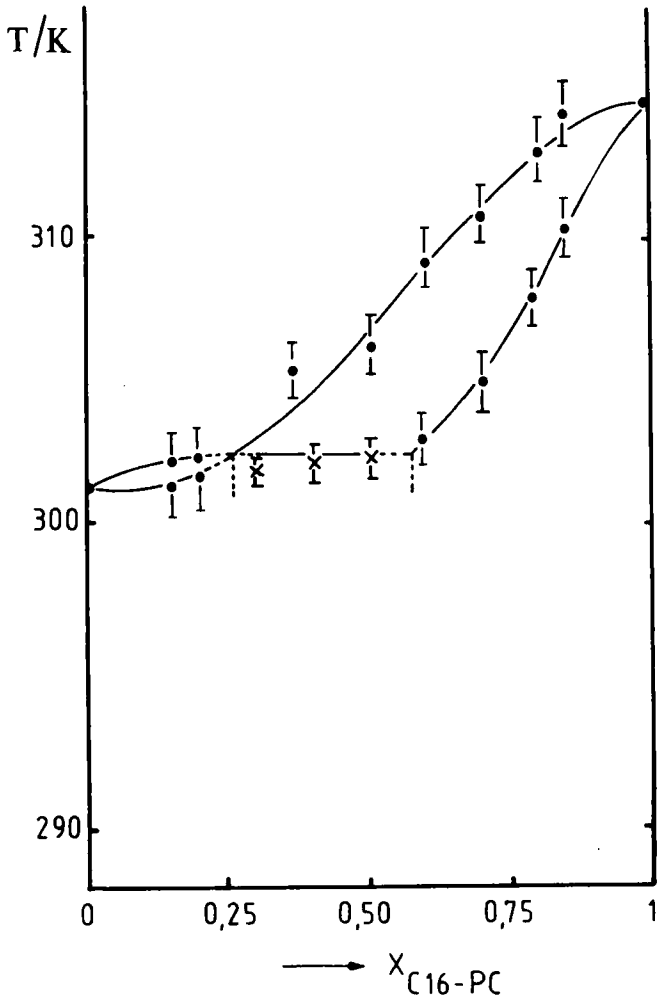



Figure 9. Phase diagram of the pseudo-binary system dipalmitoyl lecithin/di-$C_1$-methyl-palmitoyl lecithin (50 wt % water) without anaesthetics. The mole fraction is related to the dipalmitoyl lecithin concentration.

On adding heptacaine, the two lecithins become completely miscible within the high temperature phase, as proved by the solidus and liquidus curve. The low temperature phase has a miscibility gap from $x = 0{\cdot}15$ to $0{\cdot}65$. This miscibility gap has already been found for the ternary system di-(C16 : 0)-PC/di-($2C_1$-C16 : 0)-PC/$H_2O$ without added heptacaine (see §3.3). It is also confirmed by the concentration dependence of the transition enthalpies shown in figure 12 which are positive within the miscibility gap. The maximum deviation of the $\Delta\bar{H}_U$ from additivity is about 5 kJ $mol^{-1}$ and considerably higher than that of the binary system di-(C16 : 0)-PC/di-($2C_1$-C16 : 0)-PC/$H_2O$ without added heptacaine (see figure 10). Summarizing we can state that the addition of HK effects only a qualitative change within the regions of the phase diagram shown in figure 11. The melting intervals in the phase diagram at $x = 0$ and 1 are caused by the mixing properties of both systems di-(C16 : 0)-PC/$H_2O$ and di-($2C_1$-C16 : 0)-PC/$H_2O$ with heptacaine.

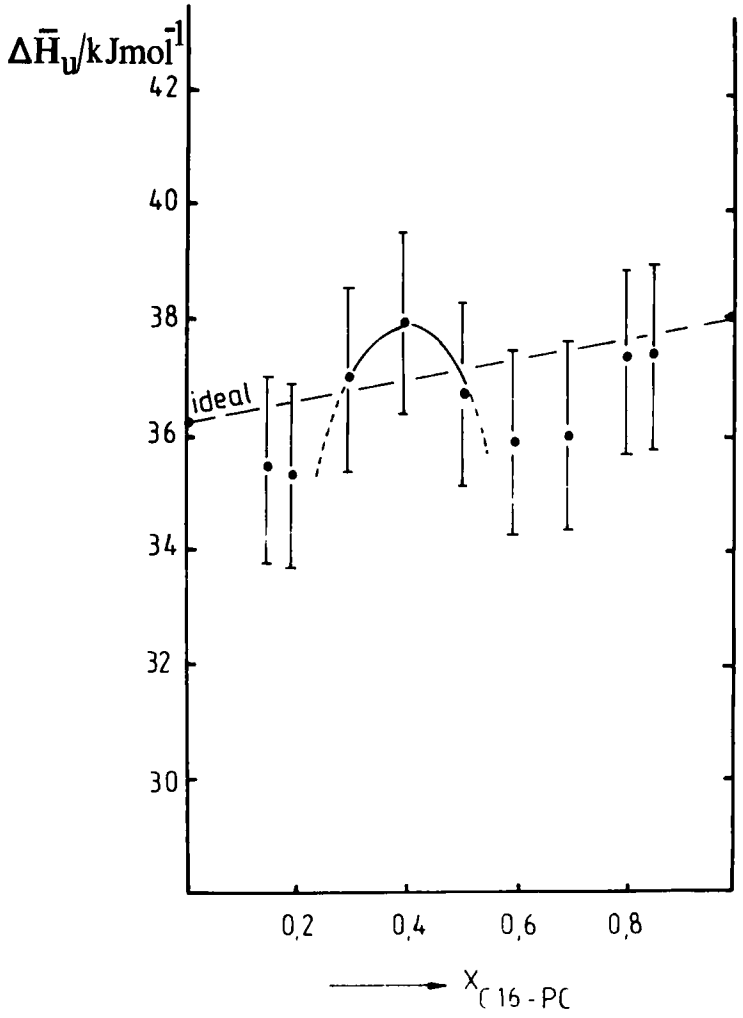



Figure 10. Phase transition enthalpies $\Delta\bar{H}_U$ (main transition) of mixtures in the pseudo-binary system dipalmitoyl lecithin/di-$C_1$-methyl-palmitoyl lecithin (50 wt % water). The mole fraction is related to the palmitoyl lecithin concentration and the enthalpies $\Delta\bar{H}_U$ of the mixtures to the water free state.

## 4. Discussion

The calorimetric experiments, presented here, demonstrate that both anaesthetics, HK and IR-1, differ in their effects when they are added to aqueous dispersions of lecithins. Incorporation of HK into the bilayer from aqueous lecithin dispersion effects a decrease in the main transition temperature. For IR-1, this decrease in the final main transition temperature is considerably smaller.

In accord with the phase diagram of the ternary system di-(C16 : 0)-PC/HK/$H_2O$, demixing occurs in the low temperature phase at mixing ratios $n_r$ of 16/1. However, if IR-1 is added instead of HK, demixing in the gel phase does not take place until the mixing ratio has reached the value 1/1.

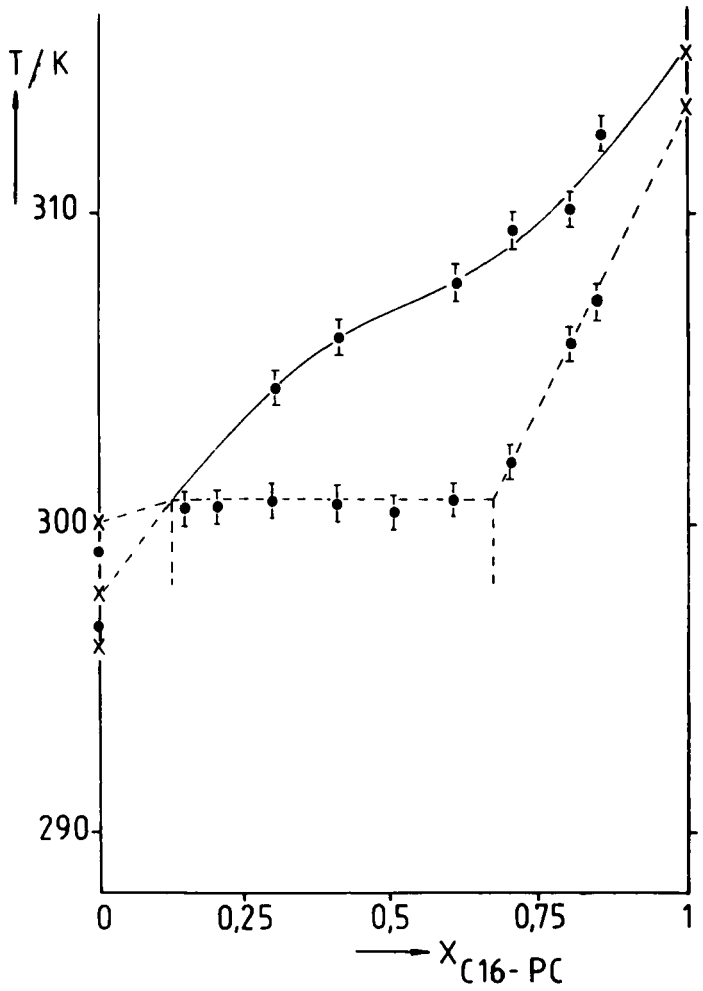



Figure 11. Phase diagram for the pseudo-binary system dipalmitoyl lecithin/di-$C_1$-methyl-palmitoyl lecithin/anaesthetic HK/water in the presence of excess water (50 wt %) and at constant IR-1 concentration ($n_r$ = 36/1). The mole fraction $x_{C16\text{-}PC}$ is related to the dipalmitoyl lecithin concentration.

Our results concerning the changes of the hydration number of the phospholipid head group by addition of anaesthetics is in accord with other results published by Cevc [42] and by Fineau and Hutchinson [43]. Cevc discussed the regulation of chain melting properties by the polar surface of the bilayer. The interfacial hydration provides, apart from the lipid chains, the most important source of the regulation of the lipid and membrane phase behaviour. The membrane properties are, therefore, strongly sensitive to the properties and characteristics of the bilayer/solution interface. Furthermore heptacaine affects the miscibility behaviour of the ternary system di-(C16 : 0)-PC/di-(2$C_1$-C16 : 0)-PC/$H_2O$. At a molar mixing ratio $n_r$ of 36/1, the miscibility gap of the phase diagram within the gel phase is extended, but the type of phase diagram is not changed. The results of the present study confirm that both anaesthetics were incorporated in the lecithin bilayers. This incorporation changes the hydration properties in the aqueous bilayers and their miscibility properties.

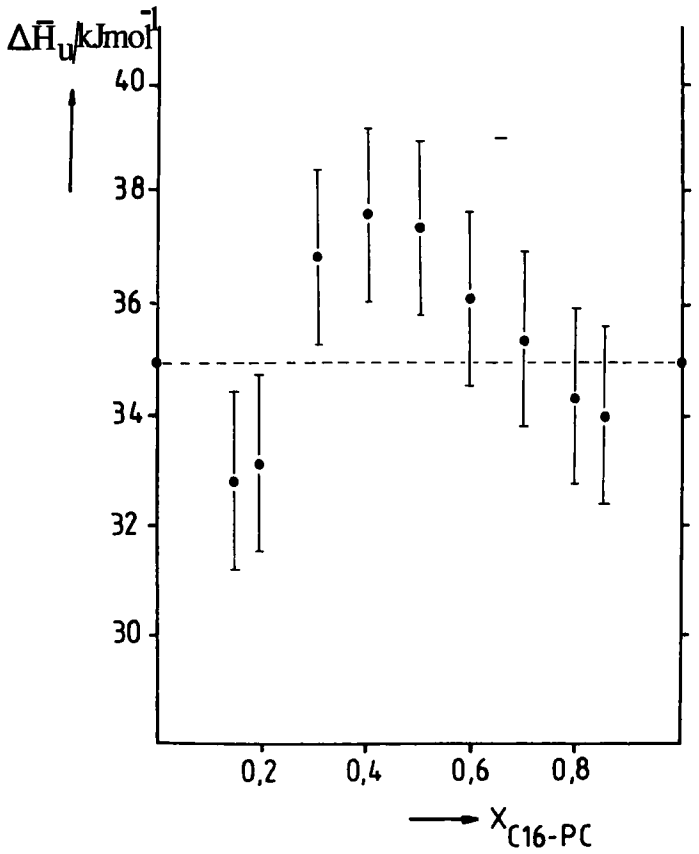



Figure 12. Concentration dependence of the transition enthaplies $\Delta\bar{H}_U$ (main transition) for mixtures in the quaternary system dipalmitoyl lecithin/di-$C_1$-methyl-palmitoyl lecithin/ anaesthetic HK/water in the presence of excess water (50 wt %) and at constant IR-1 concentration ($n_r$ = 36/1). The mole fraction $x_{C16\text{-}PC}$ is related to the dipalmitoyl lecithin concentration. The $\Delta\bar{H}_U$ values are related to the water free state.



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