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Disturbing effect of perdeuterated fatty acids used as probes in phospholipid monolayers

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Surface pressure-area per molecule isotherms have been obtained for tetradecanoic acid ($C_{14}H$) and perdeuterated tetradecanoic acid ($C_{14}{}^2H$) and their mixtures at air/water interface. The perdeuterated fatty acid was then used as a probe to evaluate the consequent disturbing effect of perdeuteration in dimyristoyl (DMPC) and dipalmitoyl (DPPC) phosphatidylcholine monolayers used as model membranes. It appears from the analysis of the transition pressure variation versus mole fraction of the probe that $C_{14}H/C_{14}{}^2H$ mixtures behave ideally, whereas mixtures of DMPC-$C_{14}{}^2H$ or DMPC-$C_{14}H$ and DPPC-$C_{14}{}^2H$ lead to negative azeotropic phase diagrams showing that the disturbing isotopic effect of the probe is really negligible with respect to the hydrocarbon chain structure as can be seen from the phase diagram analysis. According to these data, it seems that a perdeuterated lipid is suitable as a really almost non perturbing probe only if the latter constitutes the deuterated homologous of the system forming molecules under study.

Introduction

Specifically deuterium labelled or perdeuterated fatty acids and phospholipids have been used, in ^2H-NMR techniques, as molecular probes to determine the ordering of aliphatic chains in lamellar phospholipid systems [1] as well as in more complex biological membranes [2–4]. Some workers have used these probes as a tool for conformational studies of lipids using Raman spectroscopy [5]. In all these studies, the deuterated probes are generally regarded as non perturbing. Yet very few is known about the hydrogen substitution on the intrinsic conformational properties of aliphatic chains. Monolayer techniques are often used, now, as model membranes particularly in studies on interaction between the membrane components (phospholipids-cholesterol, glycolipids or fatty acids). Indeed, a generalized surface pressure-area per molecule (π-A) isotherm exhibits different regions corresponding to different monolayer phases. The socalled liquid expanded (LE)-liquid condensed (LC) transition is of high interest for membranologists since it has been related to the liquid crystalline-gel transition in bilayers [6]. For this reason, one approach to conformational problems is to study the monolayers' properties of deuterium-substituted and unsubstituted compounds. Indeed the LE-LC transition is sensitive to changes in molecular interactions, which will be indicated by a transition temperature shift. In order to get a more precise information about a possible perturbation effect in such transitions, due to the perdeuterated probes we report here a monolayer study of various bi-

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nary mixtures of $C_{14}{}^2H$ with $C_{14}H$, DMPC or DPPC.

Materials and Methods

Perdeuterated tetradecanoic acid was prepared by repeated exchange, at 240°C, in heavy water containing 4% NaO^2H, using Pd-C as a catalyser. It was purified by column chromatography using petroleum ether to eliminate perdeuterated hydrocarbons resulting from partial decarboxylation of the fatty acid. The perdeuterated fatty acid was then eluted with isopropyl ether and crystallised twice in hexane. Mass spectrometry indicated an isotopic exchange better than 96%. Tetradecanoic acid (Biochem grade) was purified by repeated crystallisation in hexane. L-α-Dimyristoyl- and dipalmitoylphosphatidylcholine, chromatographically pure, were purchased from Sigma Chemical. π-A isotherms were recorded with an automatic surface balance designed in this laboratory. Temperature was maintained constant (±0.2°C) by circulating water. Since tetradecanoic acid is slightly soluble in water at pH 2, it was spread onto a NaCl solution (1 mol·l^{-1}) used as an aqueous phase. DMPC-$C_{14}{}^2H$ and DPPC-$C_{14}{}^2H$ mixtures were dissolved in hexane/methanol (9 : 1, v/v) and spread onto the same aqueous phase.

Results and Discussion

It has been shown [7], at temperatures within the range 6–35°C, that tetradecanoic acid yields the well known isotherms which exhibit the LE-LC transition. The same type of isotherms was observed with the perdeuterated acid. Fig. 1 shows an example of the π-A isotherms of normal and perdeuterated tetradecanoic acid monolayers at 12.5 and 14.5°C. It can be noted that, at the same temperature, the perdeuterated acid exhibits, with respect to the normal one, an increase in both transition pressure and area per molecule which both increase with temperature. We shall examine first the behaviour of each acid, then binary mixtures, considering successively the area per molecule and temperature effect on surface pressure.

Pure acids

The observed increase of areas per molecule of

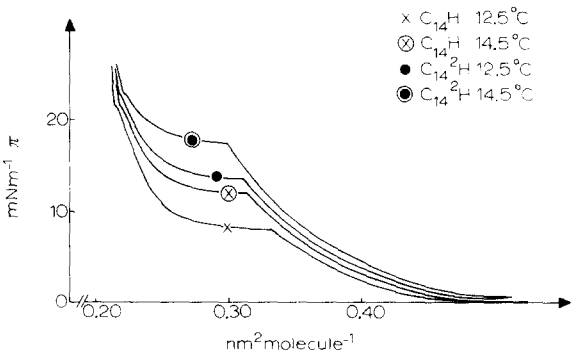

Fig. 1. Surface pressure vs. area per molecule curve of $C_{14}H$ and $C_{14}{}^2H$ monolayers at 12.5 and 14.5°C.

$C_{14}{}^2H$ with respect to $C_{14}H$ is in agreement with the data of Knoll [8], who showed, by mass densitometry and optical measurements, that the perdeuteration of fatty acid residues in DMPC lamellar phases leads to a molecular volume increase of about 30 Å^3 with respect to its hydrogenated homologous. This molecular shift, which is concomitant with a lowering of transition temperature of about 6 Cdeg, was attributed to a modification in the Van der Waals interactions or to a *gauche* rotamer formation.

Increasing temperature shifts the surface pressure to higher values with both acids. The pressure π_c corresponding to the onset of the LE-LC transition varies linearly with temperature and exhibits the same slope $d\pi_c/dT$ for both acids. In addition, a temperature shift of about 5 Cdeg is observed with the perdeuterated compound (Fig. 2). These data about such a monolayer behaviour of fatty acid chains and their deuterated homologous are consistent with those of Petersen et al. [9] who showed by differential scanning calorimetry (DSC) that the perdeuteration of fatty acid residues in phosphatidylcholine lamellar phases induces a transition temperature shift of about 5.5 Cdeg which was attributed to isotopic effects on the Van der Waals interactions between chains. Now, in a homologous series of amphiphiles, π_c varies with T according to the general expression proposed by Kellner et al. [10]

$$\pi_c = mT + b$$

where m is $d\pi_c/dT$, the Y intercept is π at $T = 0$°C and the X intercept T_0 is the lowest

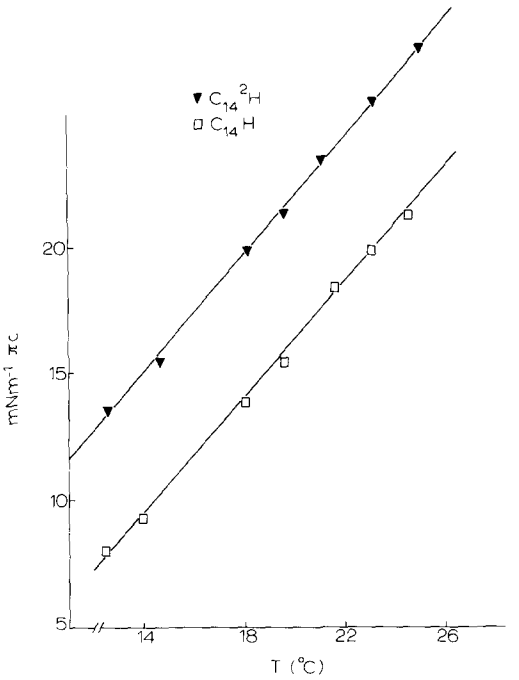

Fig. 2. π_c as a function of T for $C_{14}{}^2H$ and $C_{14}H$.

temperature at which the LE-LC transition can be detected. T_0 is of high interest since it is related to the monolayer state: the lower T_0 is, the more expanded the monolayer will be or, in equivalent terms, the shorter the chain will be. In Table I are listed the linear regression constants for the temperature dependence of the LE-LC change in monolayers of $C_{14}H$ and $C_{14}{}^2H$ from our own data and others already published in the literature dealing with $C_{14}H$. It can be noticed that the agreement is quite good. Comparison of T_0 for $C_{14}H$ and $C_{14}{}^2H$ clearly demonstrates that the deuterated acid forms a more expanded state. Thus, to evaluate the chain shortening corresponding to the observed temperature shift, we plotted T_0 versus carbon number n (Fig. 3) for the fatty acid series from our results or from literature. It is easy to note that T_0 varies almost linearly with n. Then it can be deduced that substitution of ^1H by ^2H has the same effect as a chain shortening of about 0.75 CH_2 within the accuracy of the measurement.

Thermodynamic quantities can be evaluated from the π-A isotherms. Transition heat and transition entropy can be calculated, assuming the LE-LC transition is a first order one [16], by applying the two-dimensional Clausius-Clapeyron equation modified by Motomura [17]

$$\frac{d\pi_c}{dT} = \frac{\Delta H}{T(A_e - A_c)} + \frac{d\gamma_0}{dT} \quad (1)$$

TABLE I

LINEAR REGRESSION CONSTANTS FOR THE TEMPERATURE DEPENDENCE OF THE LE-LC PHASE TRANSITION IN MONOLAYERS OF TETRADECANOIC ACID AND PERDEUTERATED TETRADECANOIC ACID

T_0, X intercept of a plot of π_c vs. T (°C); m, slope of a plot π_c vs. T ($mN \cdot m^{-1} \cdot K^{-1}$); b, Y intercept of a plot of π_c vs. T ($mN \cdot m^{-1}$); r, correlation coefficient. Substrate pH 2.

	T_0	m	b	r	Ref.
$C_{14}H$	6.8	1.17	–	–	6
$C_{14}H$	6.8	1.112	−7.58	0.998	7
$C_{14}H$	6.7	1.10	−7.3	0.997	11
$C_{14}H$	7.1	1.16	−8.2	0.994	12
$C_{14}H$	6.34	1.1967	−7.59	0.993	this study
$C_{14}{}^2H$	1.30	1.1944	−1.55	0.999	this study

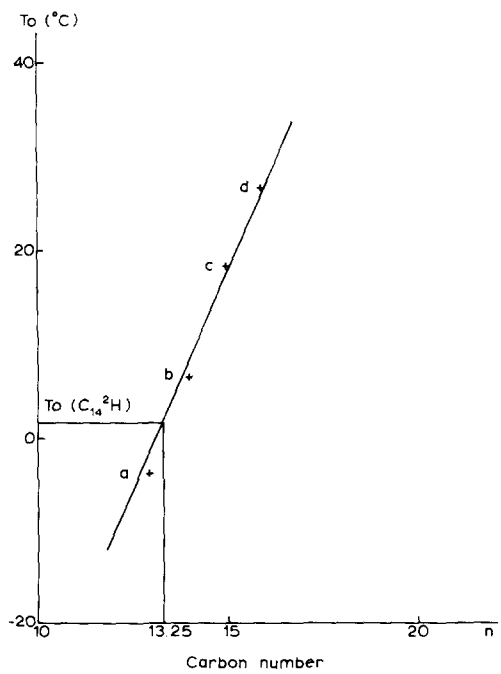

Fig. 3. T_0 as a function of carbon number n for some saturated fatty acids. a, Ref. 13; b, this work (see Table I); c, Ref. 14; d, Ref. 15.

ΔH is the heat of the phase change, A_e and A_c are the areas per molecule in the liquid expanded and condensed phases, respectively. The second term of the right hand side is related to the variation of the aqueous substrate surface tension with temperature. A_e corresponds to π_c while A_c can be either evaluated by extrapolating the condensed isotherms to $\pi = \pi_c$ or approximated according to Motomura [17] by the value of stearic acid monolayer at corresponding surface pressure. The former method was preferred to compare our own results with those of Philipps et al. [6]. Table II lists ΔH and ΔS values for normal and perdeuterated tetradecanoic acid. It can be seen that $C_{14}H$ ΔH and ΔS values calculated under the same conditions (b and c), neglecting $d\gamma_0/dT$, are in a fairly good agreement. According to Eqn. 1 the correct values, taking into account $d\gamma_0/dT$, for $C_{14}H$ and $C_{14}{}^2H$ are, respectively, 25.4 and 20.8 kJ · mol^{-1} for ΔH and 22.5 and 16.1 J · mol^{-1} · K^{-1} for ΔS at 285.5 K then it can be deduced that the material ($C_{14}{}^2H$) which has a higher transition pressure in the monolayer or a low melting temperature in the bilayer has a smaller enthalpy and a smaller entropy of transition. Now, Klump et al. [18] using DSC techniques, found larger values of transition heat and transition entropy with DPPC-d_{62} dispersions with respect to normal DPPC. This result was not expected by the authors who wrote: 'it is not obvious to us why both the entropy and the enthalpy of transition are higher for the deuterated phosphatidylcholine'. On the other hand, Cadenhead monolayers data with deuterated palmitic acid, DMPC and DPPC confirm our results [19]. Therefore $C_{14}H$ and $C_{14}{}^2H$ present different thermodynamic characteristics; it is of interest, now, to examine the behaviour of some binary mixtures.

TABLE II

HEAT AND ENTROPY CHANGES CALCULATED FOR THE TWO-DIMENSIONAL LE-LC CONDENSATION IN $C_{14}H$ AND $C_{14}{}^2H$ MONOLAYERS AT pH 2

	$\Delta H_{285.5\,K}$ (kJ · mol^{-1})	$\Delta H_{287.5\,K}$ (kJ · mol^{-1})	$\Delta S_{285.5\,K}$ (J · mol^{-1} · K^{-1})	$\Delta S_{287.5\,K}$ (J · mol^{-1} · K^{-1})
$C_{14}H$	23.4 [a]	20.9 [a]	82.0 [a]	75.4 [a]
(Ref. 6)	22.6 [b]	19.8 [b]	89.0 [b]	72.0 [b]
$C_{14}H$	22.6 [c]	20.0 [c]	79.1 [c]	65.5 [c]
(this study)	25.4 [d]	22.5 [d]	89.0 [d]	78.2 [d]
$C_{14}{}^2H$	18.5 [c]	14.3 [c]	64.8 [c]	49.7 [c]
(this study)	20.8 [d]	16.1 [d]	72.8 [d]	56.0 [d]

[a] From Ref. 6.
[b] Calculated from Ref. 6 after rectification of the slope $d\pi_c/dT$ by linear regression, neglecting d γ_0/dT.
[c] Our data, neglecting d γ_0/dT.
[d] Calculated taking into account d γ_0/dT.

Binary mixtures

In order to evaluate the miscibility of these components and to understand the eventual interactions occurring in a mixed monolayer, we systematically investigated various binary mixtures.

$C_{14}H$-$C_{14}{}^2H$ binary mixtures. Due to the very small area per molecule difference between $C_{14}H$ and $C_{14}{}^2H$, 3 Å^2, the study of miscibility out of the phase transition region, from the mean area curve, is not significant. In Fig. 4, we plotted the phase diagram that is π_c versus $C_{14}{}^2H$ mole fraction both in expanded and condensed phases according to the approach of Motomura [17].

We applied the following thermodynamic equation relative to binary mixed monolayers

$$X^{\pi,c} = X^{\pi,e} + \left[(A_c - A_e) \frac{\partial \pi^c}{\partial X^{\pi,e}} \Big/ \frac{kT}{X^{\pi,e}(1 - X^{\pi,e})} \right] \qquad (2)$$

where $X^{\pi,c}$ and $X^{\pi,e}$ are the mole fractions of the host lipid in the condensed and expanded mono-

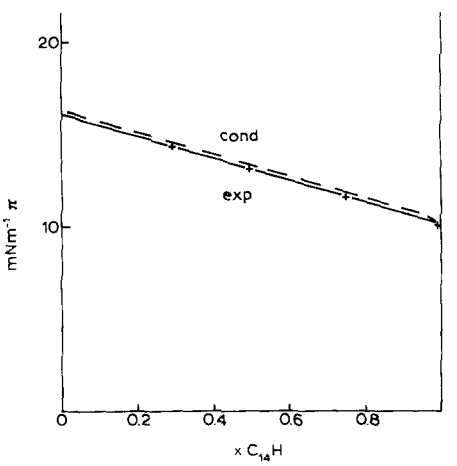

Fig. 4. Phase diagram of $C_{14}H$-$C_{14}{}^2H$ binary mixtures at 25°C.

layer, respectively, A_e is the area per molecule corresponding to the well defined onset of the pressure corresponding to the LE-LC transition, A_c was evaluated by extrapolating the condensed isotherms to $\pi = \pi^c$ and k is the Boltzman's constant.

This diagram corresponds to an almost ideal cigar type. It indicates an almost ideal mixing and a complete miscibility in the expanded as well as in the condensed state of the two forming film components. Besides, the compositions of the expanded state as well as the condensed one are practically the same at any composition of the mixed monolayer. Thus the perdeuterated acid, with respect to the normal one, appears as an almost perfect probe. Yet, at a mole fraction of the probe of about 0.1, which is the molar ratio usually used in bilayers, using NMR techniques, a slight transition pressure shift is observed corresponding to an induced temperature shift no more larger than 0.5 Cdeg. This shift is within the accuracy of DSC measurements, a fact which corroborates the suitability of such a probe.

DMPC-$C_{14}{}^2H$ binary mixtures. It is of high interest, now, to study binary mixtures of phospholipids and $C_{14}{}^2H$ since deuterated fatty acids are commonly used as probes in model membranes. DMPC was chosen for two reasons: (i) the corresponding isotherms exhibit, at the used temperature (16.5°C) the LE-LC transition as does the deuterated compound, (ii) the phospholipid chains have the same length as the deuterated probe which should maximize the interactions between the host lipid and the probe.

Fig. 5 shows π-A isotherms of DMPC-$C_{14}{}^2H$ mixed monolayers at different mole fractions. In this particular case, it is possible to investigate, through the mixed monolayers, the region within the phase transition as out of this region.

Fig. 6 shows the phase diagram of the DMPC-$C_{14}{}^2H$ mixed monolayer at 16.5°C and pH 2. Such a diagram corresponds to a negative azeotropic type which exhibits a minimum and indicates that the mutual interaction between the two components in the mixed monolayer is stronger than the interaction between the pure component molecules. In addition, the components are miscible in the expanded as well as in the condensed state. Moreover, from the phase diagram, it can be noticed that, at a given surface pressure, the mole fraction of the expanded phase is different from that of the condensed one (as indicated, for example, by points M and N at 9 $mN \cdot m^{-1}$ in Fig. 6) except at the minimum point.

Besides, it can be seen that the mixed monolayer state at 4 and 20 $mN \cdot m^{-1}$ surface pressure, denoted by arrows a and c, consists in an expanded and condensed one phase region, respectively, regardless of the monolayer's composition and simultaneously a negative deviation of area

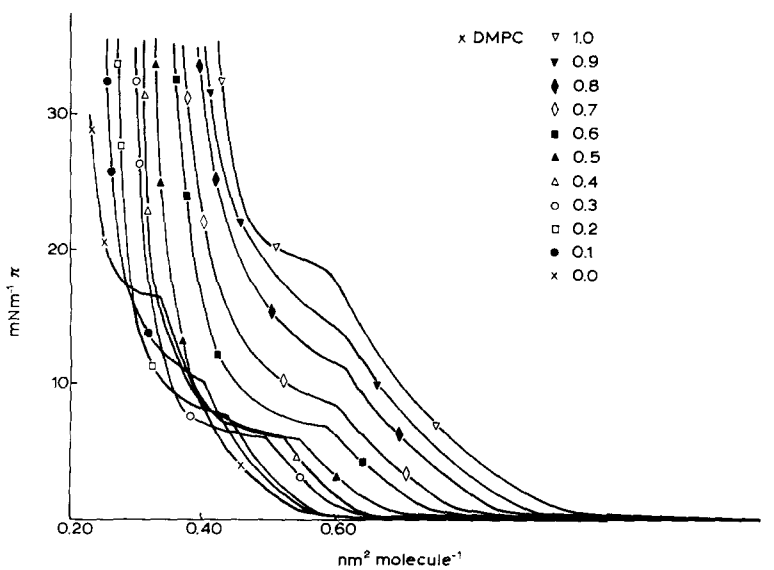

Fig. 5. Surface pressure vs. mean area curves of the DMPC-$C_{14}{}^2H$ mixed monolayer at 16.5°C. The curves were continuously recorded. The symbols are used only to designate the film composition.

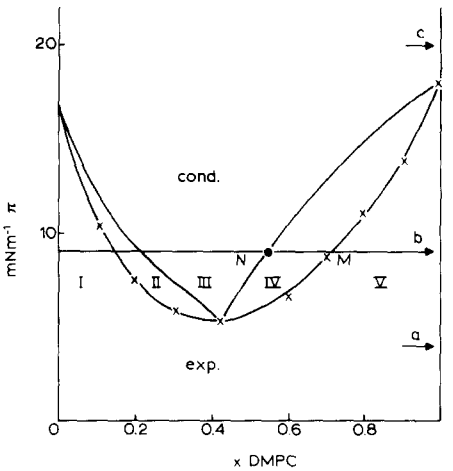

Fig. 6. Phase diagram of DMPC-$C_{14}{}^2H$ binary mixtures at 16.5°C.

per molecule is observed (curves a and c in Fig. 7) as expected from the phase diagram. Indeed, at these both surface pressures, it is noteworthy that, from a 0.2 to 0 mole fraction of the probe, the additivity rule is obeyed indicating an ideal mixing between the two film components. Nevertheless, the transition phase surface pressure of the pure host lipid is lowered by about 20% when the mole fraction of the probe is only 0.1.

The mean area curve at 9 $mN \cdot m^{-1}$ (curve b in Fig. 7) consist in five different parts. In agreement with the phase diagram of Fig. 6, an ideal expanded one-phase region is observed at very low or rather high mole fraction of the probe (v and i); a condensed one phase region, with a negative deviation is observed between 0.3 and 0.5 mole fraction of the host lipid (iii region) while the ii and iv domains consist in a two phase region.

In order to evaluate the contribution of the isotopic effect about the mixed monolayer behaviour, we investigated DMPC-$C_{14}H$ binary mixtures phase diagram (Fig. 8).

As can be seen, it corresponds to a negative azeotropic type similar to DMPC-$C_{14}{}^2H$ one and the minimum point in the two phase diagrams occurs at the same film composition. Besides, the right-hand branches of two phase diagrams are practically identical till a mole fraction of the host lipid of about 0.7. Moreover, due to the more expanded state of the deuterated fatty acid, the left-hand branches of the DMPC-$C_{14}{}^2H$ phase diagram are shifted to higher surface pressures with respect to the DMPC-$C_{14}H$ ones. As the probe is commonly introduced in the host lipid in a mole fraction of about 0.1 it can be deduced that

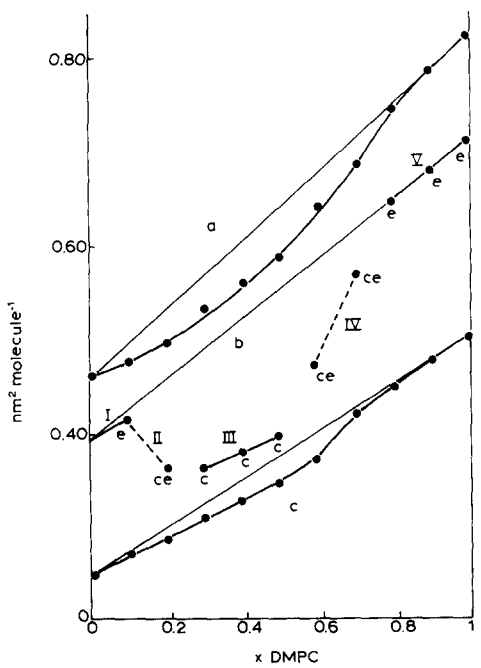

Fig. 7. Mean area per molecule as a function of composition for the system DMPC-$C_{14}{}^2H$ at 16.5°C.

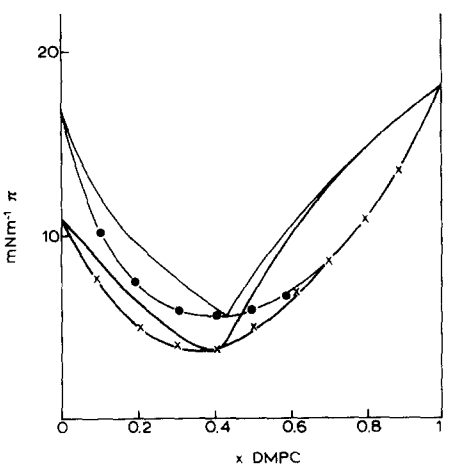

Fig. 8. Phase diagram of DMPC-$C_{14}H$ binary mixtures at 16.5°C (×) compared to phase diagram of DMPC-$C_{14}{}^2H$ binary mixtures at 16.5°C (●).

the disturbing effect is due, not really to deuterium, but essentially to the nature of the probe, that is to the structure of the hydrocarbon chains.

DPPC-$C_{14}{}^2H$ mixtures. In this case, we investigated the influence of the probe when the latter does not present the same chain length as the host lipid acyl chains. At 25°C, DPPC LE-LC transition occurs at a much lower surface pressure than that of the pure probe. The phase diagram is similar to the previous one, that is, a negative azeotropic type (Fig. 9). The surface pressure shift induced by the presence of the probe is similar but, due to the difference in the transition pressures of the pure compounds, a second condensed phase occurs only at high mole fractions of the deuterated component, which obviously is not the normal condition of probe use. The area per molecule-mole fraction curves (not represented here) are rather complex. Nevertheless it can be deduced that at any surface pressure, the expanded phase, corresponding to the usual probe content, deviates from ideality, which proves the existence of some interaction between the host lipid and the probe. It is reasonable to assume that in this case as in the previous one, the disturbing effect is not due to deuterium but essentially to the nature of the hydrocarbon chains.

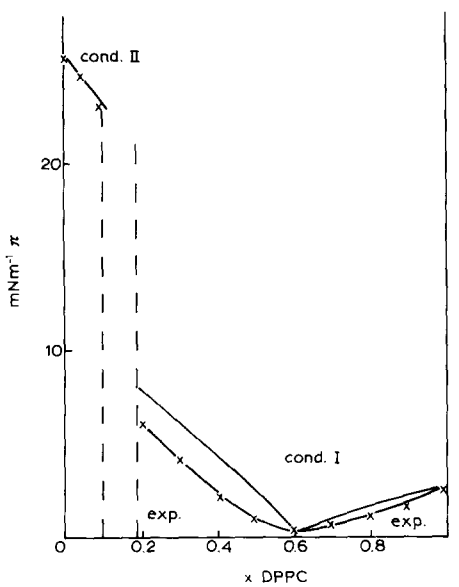

Fig. 9. Phase diagram of DPPC-$C_{14}{}^2H$ binary mixtures at 25°C.

Conclusion

In the present work, attention was focused on the consequent disturbing effect of the use of deuterated probes. This effect has been investigated through the study of different binary mixtures such as $C_{14}{}^2H$ and $C_{14}H$, DMPC or DPPC. It has been shown that the $C_{14}H$-$C_{14}{}^2H$ system behaves almost ideally while the $C_{14}{}^2H$-DMPC and $C_{14}{}^2H$-DPPC systems lead to azeotropic phase diagrams, indicating obvious interactions between the host lipid and the probe. Through the phase diagram analysis of DMPC-$C_{14}H$ and DMPC-$C_{14}{}^2H$ binary mixtures it appears that the observed perturbation is essentially related not to an isotopic effect but to the hydrocarbon chain structure of the probe.

The most widely used probes in the study of membrane organization and dynamics are fluorescent probes and spin label probes. The disturbing effect of fluorescent probes was investigated by Lösche [20] while Cadenhead [21] showed that tetradecanoic acid isotherms are strongly modified when 1% of 12-doxylstearic acid is introduced in the monolayer. With deuterated probes, it could be expected that, due to the large similarities of deuterium substituted and unsubstituted compounds, this perturbing effect would be non existent. The present data show that it is the case only if the host lipid and the probe have the same chain structure. Then, it must be kept in mind that deuterated probes are not systematically suitable, contrary to a wide spread idea that, due to the negligible steric hindrance of deuterium, they induce no perturbation in the systems under study. In addition, great care must be exercised in interpreting results since even in expanded phase, interactions would occur between the host lipid and the probe.

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