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Chiral discrimination in *N*-tetradecanoylalanine and *N*-tetradecanoylalanine / ditetradecanoylphosphatidylcholine monolayers

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Surface pressure-area per molecule isotherms have been obtained for *N*-tetradecanoyl-L-alanine (myristoyl-L-alanine, L-MA), *N*-myristoyl-D-alanine (D-MA) and their mixtures at the air / water interface at various temperatures to detect an eventual chiral discrimination occurring in *N*-acylamino-acid monolayers. It was found that the racemic mixture D-MA + L-MA exhibits, at a given temperature, a more expanded state than that of the pure enantiomeric one. Besides, the phase diagram of L-MA / D-MA binary mixtures gives a positive azeotropy which means that the interactions between the two enantiomeric molecules (L–D) are weaker than the interactions between the pure chiral molecules (L–L or D–D), these interactions being at their minimum level when the mixture corresponds to the racemic composition. Any measurable difference in the interactions between two different chiral molecules is referred to as a diastereoisomer discrimination, compared to an enantiomer discrimination between two antipodes. This was checked through L-dimyristoylphosphatidylcholine L-DMPC) / L- and D-MA mixed monolayer studies. No difference was observed in the phase diagram of binary mixtures of L-DMPC / L-MA and L-DMPC / D-MA, which shows that, in such systems, diastereoisomer interactions cannot occur because of the relative inaccessibility of the phospholipid chiral center.

Introduction

Chiral properties of lipid monolayers at air/water interfaces have received little attention, although their biophysical and biological implications could be important. Indeed, the major components of cell membranes such as phospholipids, sterols and proteins contain chiral centers which are involved in the stereochemistry of interactions determining the organization and dynamics of these membranes.

However, this aspect has been more or less ignored up to now and the few key studies of chiral interactions in monolayers are those reported by Lundquist [1–3], who examined the monolayer behaviour of the optically pure enantiomer of tetracosan-2-ol and its acetate derivates in relation to their racemic form, and showed that it was possible to demonstrate a chiral discrimination between the pure enantiomers and the racemic mixtures. Tachibana et al. [4] showed an enantiomer discrimination involving monolayers of racemic D,L and L-12-hydroxystearic acid, and found that the racemic compound leads to a pressure/area per molecule curve (π-A) which indicates a more expanded state in the compressible

Abbreviations: L-DMPC, dimyristoylphosphatidylcholine; L-MA, *N*-myristoyl-L-alanine; D-MA, *N*-myristoyl-D-alanine, LE, liquid expanded; LC, liquid condensed

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region. More recently, Arnett et al. demonstrated an acid-dependent chiral discrimination involving monolayers of the enantiomeric and the racemic form of *N*-α-methylbenzylstearamides, which was described in some short communications [5,6] but failed to prove a significant difference between racemic dipalmitoylphosphatidylcholine and its enantiomers.

N-Acylamino acids containing one or two chiral centers in their polar headgroups appear to be suitable amphiphiles for investigating the stereochemical factors determining the monolayer properties. They offer a large variety of polar headgroups which can be modified in a further step to constitute peptides containing more chiral centers. Such compounds are also interesting models for investigating the interactions between peptide residues and phospholipid polar headgroups which are involved in the interactions of numerous peptides, such as hormones, enkephalins and antibiotics with biological membranes.

For these reasons, we report here a monolayer study of L-MA and L-MA/D-MA binary mixtures through which a striking chiral discrimination is observed.

Materials and Methods

L-MA and D-MA were prepared by coupling the amino-acid sodium salt with myristoyl chloride as an acyl donor. The reaction solvent was acetone/water, 1:1, and the chloride was dissolved in dry acetone and added dropwise to the reaction solution, which was vigorously stirred. After acidification, the precipitate was isolated by filtration, dried and exhaustively washed with pentane to remove myristic acid. The *N*-acylamino acid was crystallized in hexane/ethanol (9:1, v/v) and chromatographed on a column of silica gel using chloroform/ethanol (1:1, v/v) as eluent.

The enantiomeric acyl-amino acids were identified by mass spectrometry ($M^{+}299$) and infrared characteristic bands in solid state: ν_{N-H}, 3270 cm^{-1}; $\nu_{C=O}$(COOH), 1740 cm^{-1}; amide band I, 1625 cm^{-1}; amide band II, 1562 cm^{-1}.

Specific rotations were, in ethanol

$[\alpha]_D^{20} = +45.8 \pm 0.8° \cdot mol^{-1} \cdot l \cdot m^{-1}$ for L-MA

$= -46.2 \pm 0.8° \cdot mol^{-1} \cdot l \cdot m^{-1}$ for D-MA

Chromatographically pure L-α-dimyristoylphosphatidylcholine was purchased from Sigma. It was crystallised twice in acetone/methanol, 95:5 (v/v). Surface pressure (π)/area per molecule (A) isotherms were recorded using an automatic surface balance designed in this laboratory. A constant temperature was maintained ($\pm 0.2°$ C) by circulating water. The compounds were spread from a hexane/ethanol (9:1) solution onto a roasted NaCl ($1 \text{ mol} \cdot l^{-1}$) solution, at pH 2, due to their slight solubility in water.

Results and Discussion

I. Enantiomeric and racemic monolayer behaviour

In Fig. 1 are recorded the π-A isotherms of L-MA, D-MA and racemic DL-MA at 18° C. These isotherms exhibit the so-called liquid-expanded (LE) – liquid-condensed (LC) transition which is sensitive to molecular interactions and temperature (see below). It can be noticed that, for symmetry reasons, L- and D-MA exhibit the same isotherms characterized by a transition pressure at 1.4 $mN \cdot m^{-1}$ while the racemic mixture gives a higher transition surface pressure equal to 12.8 $mN \cdot m^{-1}$. Moreover, the areas per molecule, practically identical at low surface pressure, are shifted, in the condensed region, to rather higher values with the racemic mixture. Thus, a clear enantiomer discrimination is shown through these monolayers.

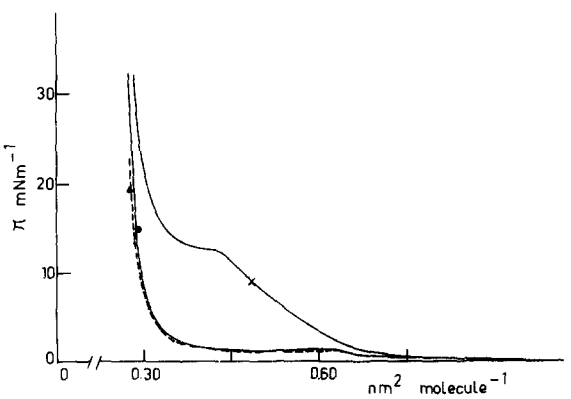

Fig. 1. Surface pressure vs. area per molecule curve of L-MA (●), D-MA (▲) and racemic D,L-MA (×) monolayers at 18° C. (Subphase: pH 2, NaCl: 1 M).

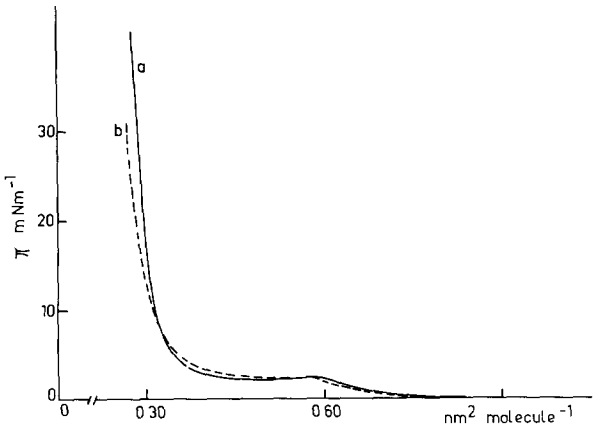

Fig. 2. Surface pressure vs. area per molecule curve of L-MA monolayers at 19.5°, at two compression rates: (a) 0.10 $nm^2 \cdot mol^{-1} \cdot min^{-1}$; (b) 0.02 $nm^2 \cdot mol^{-1} \cdot min^{-1}$.

Such chiral films are very much affected by the kinetic effect: in Fig. 2 are plotted L-MA isotherms at two compression rates (0.1 and 0.02 $nm^2 \cdot mol^{-1} \cdot min^{-1}$). It can be noticed that, at rapid compression (curve a), for L-MA isotherm, the onset of the LE–LC transition pressure exhibits a maximum corresponding to a non-equilibrium state (characterized by a hump in the isotherm). Moreover, the intersection of the two isotherms indicates some relaxation of the film at low compression rate. This is not observed with myristic acid monolayers which lead to practically the same isotherms whatever the compression rate be. According to these results, it may be concluded that the reorganization of chiral polar headgroups during the monolayer relaxation is really a slow process, whereas the carboxylic-group reorientation seems to be much less affected by the compression rates used. Such behaviour about chiral monolayers was reported by Thompson [7,21] who showed, through monolayers studies, that optically pure *N*-α-benzylstearamide films exhibit a high sensibility towards kinetic factors, and observed the same metastability as ours in the π-*A* curve at the onset of the LE–LC transition. All the isotherms were recorded at a compression rate of 0.01 $nm^2 \cdot mol^{-1} \cdot min^{-1}$.

The chiral discrimination can also be proved quantitatively through the dependence of π_c on temperature. Indeed, it is well known, that for most of the long chain amphiphiles which exhibit

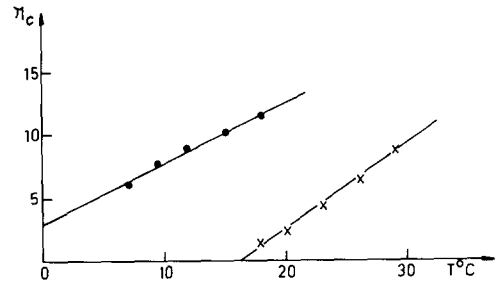

Fig. 3. π_c as a function of T for L-MA (×) and D,L-MA (●).

an LE–LC transition, π_c increases with increasing temperatures. Plots of π_c versus T for L-MA and D,L-MA give straight lines, as shown in Fig. 3, the slopes (0.771 and 0.445 $mN \cdot m^{-1} \cdot K^{-1}$, respectively for L-MA and D,L-MA) are different. The x-intercepts T_0, the lowest temperatures at which the LE–LC transition can be detected, for both monolayers, are also different.

Comparison of the T_0 values clearly demonstrates that the racemic monolayer ($T_0 = -5.9\,°C$) gives a more expanded state than that of the pure enantiomeric one ($T_0 = +16.9\,°C$).

What is the meaning of the observed difference?

Usually π_c varies with T according to the general expression proposed by Kellner et al. [8]:

$$\pi_c = mT + b,$$

where m is $d\pi_c/dT$.

Now, in fatty-acid series, for a given subphase (HCl, pH 2), m is practically constant within the accuracy of measurements; 1.24 for tridecanoic acid [9], 1.19 for tetradecanoic acid [10]; 1.21 for pentadecanoic acid [11] and 1.12 for hexadecanoic acid [12], so that the average value of m is 1.19 ± 0.05 $mN \cdot m^{-1} \cdot K^{-1}$. Attempts to generalize the constancy of m to homologous series of other amphiphiles were not fruitful because of the great dispersion of m values through literature. As an example, were found for ethyl hexanoate, m values of 0.68 [11] and 0.45 $mN \cdot m^{-1} \cdot K^{-1}$ [13] at pH 2. This may be due to the degree of purity of the samples used. Indeed, the purity effect is of most importance on the LE–LC transition pressure values and consequently on the m values. As an example, the introduction of 5% perdeuterated myristic acid in a normal myristic acid monolayer

increases π_c by about 10% [10]. Thus, it could be suggested that m characterizes the polar headgroup.

Thermodynamic quantities can be evaluated from the π-A isotherms. Transition heat ΔH and transition entropy ΔS can be calculated, assuming that the LE–LC transition is a first order one [14], by applying the two-dimensional Clausius-Clapeyron equation modified by Motomura [15]

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

A_e and A_c are the transition areas per molecule in the liquid-expanded and the liquid-condensed phases, respectively. The second term on the right handside is related to the variation of the aqueous substrate surface tension with temperature. A_e corresponds to the well-defined onset of the LE–LC transition pressure, while A_c was evaluated by extrapolating the condensed isotherms to $\pi = \pi_c$.

Table I lists ΔH and ΔS values for L-MA and D,L-MA at 18°C. It can be noted that the racemic material which has a higher transition pressure in the monolayer has a smaller enthalpy, 7.9 ± 0.6 kJ · mol^{-1} (44 ± 3 kJ · mol^{-1} for L-MA) and a much smaller entropy, 27 ± 3 J · mol^{-1} · K^{-1} (150 ± 7 J · mol^{-1} · K^{-1} for L-MA) of transition. In the light of these data and according to the suggestion of one of the referees, it would seem that the more expanded condensed region can be explained by poorer packing of the D,L-form and that the less expanded expanded state is due to the same poor packing permitting the chains to approach each other closer, before the intermolecular forces are strong enough to bring about the transition.

TABLE I

TRANSITION HEAT AND TRANSITION ENTROPY FOR L-MA AND RACEMIC D,L-MA AT 291 K, CALCULATED USING THE TWO-DIMENSIONAL CLAUSIUS-CLAPEYRON EQUATION

	ΔH (kJ · mol^{-1})	ΔS (J · mol^{-1} · K^{-1})
L-MA	44 ±3	150±7
D,L-MA	7.9±0.6	27±3

II. Binary mixtures

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

L-MA and D-MA binary mixtures. Fig. 4 represents the π-A curves of mixed monolayers of various compositions. Mean area per molecule (A) vs. mole fraction (X) curves are of large use now, when attempting to obtain information on molecular packing, miscibility and interactions in mixed monolayers [16], but, as it can be noticed, in Fig. 4, due to the small mean area per molecule variation with the film composition in the expanded as well in the condensed region, the study on A-X curves is not significant. Indeed, in the expanded state, as an example, the difference between the area per molecule at 0 and 0.5 mole fraction is about 0.03 nm^2 · mol^{-1} at 1 mN · m^{-1} surface pressure. In this interval, about ten isotherms were plotted, which implies an accuracy of about 0.003 nm^2 · mol^{-1} when the experimental accuracy is only 0.005 nm^2 · mol^{-1}. On the other hand, an important shift to higher transition pressure is observed within 0.1 mole fraction of D-MA in the mixed monolayer. As the D-enantiomer mole fraction increases, the transition pressure increases, indicating a more and more expanded film as the binary mixture tends to the racemic composition. It is worth noting that, between 0.15 and 0.4 D-enantiomer mole fraction, the isotherms exhibit another transition pressure which varies with the

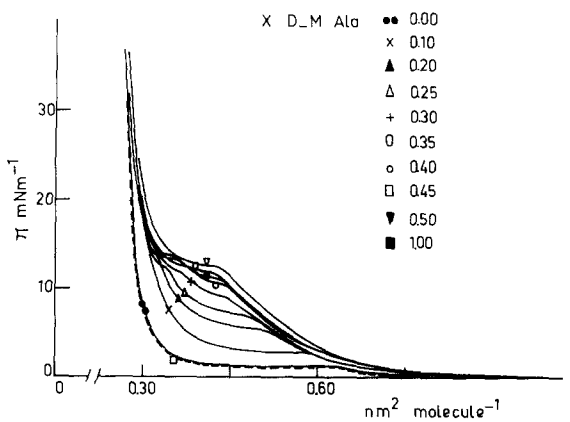

Fig. 4. Surface pressure vs. mean area curves of the L-MA/D-MA mixed monolayer at 18°C.

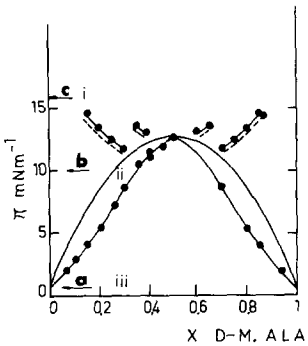

Fig. 5. Phase diagram of L-MA/D,L-MA binary mixtures at 18 °C. Arrows a and c: expanded and condensed one-phase region, respectively; arrow b, mixed monolayer consists of three different states according to the mole fraction: (i) condensed one-phase region; (ii) two-phase region; (iii) expanded one-phase region.

film composition, whatever the compression rate. Thus, some relaxation phenomena seem to be excluded. This second transition should lead to a miscible second condensed phase (C_2) since its surface pressure varies with the film composition, as observed in Fig. 4. From the phase rule, it can be deduced that the system does not belong to the eutectic type (with phase separation) [17].

At the transition plateau of the π-A curve, the expanded monolayer is in equilibrium with the condensed one. When two components are miscible in both the expanded and condensed states, the mole fraction of the component in the condensed monolayer can be obtained according to Motomura's approach [15]. It is then possible to obtain for each transition two curves; one is the experimental curve $\pi_{c1} - X$ and the other can be calculated using the following equation:

$$X^{\pi_{c1}} = X^{\pi_e} + \frac{(A_{c1} - A_e) \cdot \delta\pi_{c1}/\delta X^{\pi e}}{kT/X^{\pi_e}(1 - X^{\pi_e})}$$

$X^{\pi_{c1}}$ and X^{π_e} are the mole fractions of D-MA in the condensed and in the expanded state, respectively, A_e and A_c are the transition area values in the liquid-expanded and the liquid-condensed phases, respectively, and k is the Boltzman constant. The same approach gives the two curves related to the second transition, $C_1 \rightarrow C_2$.

Fig. 5 represents the two-dimensional phase diagram of L-MA/D-MA system that is the composition of the expanded and condensed phases in equilibrium for a given surface pressure. This phase diagram is characterized by a maximum point which corresponds exactly to the racemic composition. Thus this point may be called a two-dimensional positive azeotropic point. This behaviour indicates that the mutual interactions between the two antipodes in the monolayer are weaker than the interactions between the pure enantiomeric molecules themselves. Besides, it can be seen that the mixed monolayer state at 0.5 and 16 mN · m^{-1} surface pressure denoted by arrows a and c consists of an expanded and condensed one-phase region, respectively, regardless of the monolayer's composition. At 10 mN · m^{-1} surface pressure, denoted by arrow b, the mixed monolayer consists of three different parts according to the mole fraction: a condensed one-phase region (i); an expanded one-phase region (iii), and a two-phase region (ii).

The second transition pressure variation with film composition resembles a branch of a negative azeotropic type between 0.15 and 0.3 mole fraction of the D-enantiomer but beyond 0.3 mole fraction, the variation of the transition surface pressure with the film composition does not correspond to any known type of phase diagram as described by Matuo et al. [18]. Further studies must be undertaken to clear it. The origin of this second transition could be due to particular interactions involving amino acid residues: steric hindrance and flexibility of the polar headgroup which might lead to different conformations.

L-DMPC/L-MA and L-DMPC/D-MA binary mixtures. Diastereoisomer discrimination is the ability of a chiral molecule to distinguish between the enantiomers of another chiral molecule. In order to detect an eventual diastereoisomer interaction occurring between L-DMPC and L-MA or D-MA, we investigated L-DMPC/L-MA and L-DMPC/D-MA binary mixtures. DMPC was chosen for two reasons: (i) the corresponding isotherms exhibit, at the used temperature (≈ 20 °C) the LE–LC transition, as does L-MA; (ii) the phospholipid chains have the same length as that of the lipoamino acid, which should maximize the interactions between the two components.

Fig. 6 shows π-A isotherms of L-DMPC/L-MA at different mole fractions. In this particular case,

it is possible to investigate, through the mixed monolayer, the region within the phase transition as out of this region. Fig. 7 shows the phase diagram of the L-DMPC/L-MA mixed monolayer at 19.5 °C and pH 2. From 0.3 to 1 mole fraction, this diagram corresponds to a negative azeotropic type which exhibits a minimum point at 0.6 mole fraction of the phospholipid. It indicates, in this mole fraction range, that the components are miscible in the expanded as well as in the condensed state, and the mutual interaction between the two components in the mixed monolayer is stronger than the interaction between the pure components themselves.

From 0 to 0.2 L-DMPC mole fraction, a second branch is observed, separated by a domain in which the transition is rather soft. As can be seen, the mixed monolayer state at 1 and 30 $mN \cdot m^{-1}$ surface pressure, as denoted in Fig. 7 by arrows c and a, consists in an expanded and a condensed one-phase region, respectively, regardless of the monolayer's composition, and simultaneously a negative deviation of mean area per molecule, with respect to additivity, is observed (curves c and a in Fig. 8) as expected from the phase diagram. It is interesting to note that, between the transition surface pressures of the pure components, i.e., at 10 $mN \cdot m^{-1}$, the mean area curve presents a break (dashed area in Fig. 8) as does the phase diagram. Indeed, the monolayer state, at this surface pressure, varies with the film composition (i) a condensed one-phase region between 0 and 0.15 L-DMPC mole fraction, (ii) and expanded one-phase region between 0.3 and 1 mole fraction separated from the former by a break which is probably due to the large difference between the LE–LC transition surface pressure of the pure components' monolayers as already observed with L-dipalmitoylphosphatidylcholine/perdeuterated tetradecanoic acid system [10]. No significant difference was observed in the phase diagram and the mean area curves between L-DMPC/L-MA and L-DMPC/D-MA systems beyond the errors of the techniques, then diastereoisomer discrimination cannot be demonstrated in such systems. According to these results, it may be concluded that polar interaction between the phospholipid and the lipoamino acid does not involve the two chiral sites but the amino acid and the phosphocholine residue.

Attempts to detect diastereoisomer interactions in mixed monolayers of cholesterol with enanti-

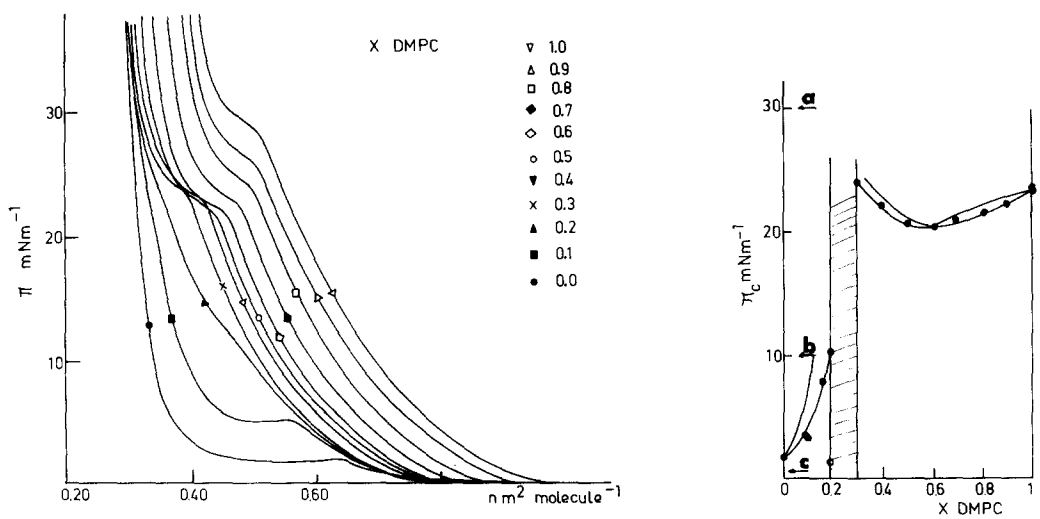

Fig. 6. Surface pressure vs. mean area curves of the L-DMPC/L-MA mixed monolayer at 19.5 °C.

Fig. 7. Phase diagram of L-DMPC/L-MA binary mixtures at 19.5 °C. Arrows a (30 $mN \cdot m^{-1}$) and c (1 $mN \cdot m^{-1}$) condensed and expanded one-phase region, respectively; arrow b (10 $mN \cdot m^{-1}$): according to the mole fraction, mixed monolayer consists of either the one-or the two-phase region.

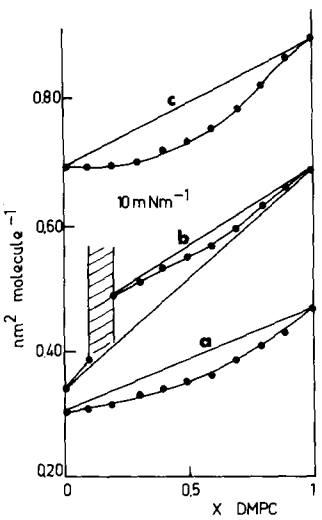

Fig. 8. Mean area per molecule as a function of composition for the system L-DMPC/L-MA at 19.5 °C. a, b and c correspond to the surface pressures 30, 10 and 1 mN·m^{-1}, respectively.

omeric phospholipids were reported by Ghosh et al. [19]. As in our case, no difference was revealed in the behaviour of the mixed systems. Thus, the relevance of such data to the role of this chiral center remains obscure.

General Discussion

Variation of π_c with the composition (L/D) of a mixed monolayer has never been reported. Thus, bidimensional phase diagrams of chiral monolayers were never described. Before discussing the different aspects of chiral discrimination occurring in monolayers, it is instructive to examine, as a comparison, the properties of mixtures of enantiomers in the solid state. Three fundamental types of racemates may be defined on the basis of a melting phase diagram [20] (i) the conglomerate (or eutectic); (ii) the racemic compound in which the two enantiomers coexist in the same unit cell; (iii) the solid solution in which the two enantiomers form mixed crystals. We show, here, that the systems studied exhibit a behaviour similar to that observed in solid solutions. As can be seen in Fig. 1, chiral discrimination is not evident along the LE region since the areas per molecule are practically identical with the enantiomers and the racemic mixture monolayers. This is consistent with the thermodynamic treatment done by Shröder and reported in Ref. 20 who showed that mixtures of antipodes behave ideally in the liquid state. Thus, if both the racemic mixture and the pure enantiomer give the LE state, the π-A isotherms might be identical until the monolayer reaches the LE–LC transition. On the other hand, chiral discrimination occurs at the level of the transition plateau: indeed, Fig. 5 shows a positive azeotropic phase diagram (similar to that exhibited by a solid solution), which means that homochiral interactions (L–L) are stronger than heterochiral ones (L–D).

If our results are comparable to those of Lundquist [2] and Arnett [5], they really differ from those of Tachibana et al. [4], who reported a well-defined example of an enantiomer discrimination involving D and D,L-12-hydroxystearic acid (where the chiral center is located in the hydrocarbon chain). Their data showed that the racemic film exhibits a higher transition pressure and a larger area per molecule in the LE region than that of the optically active forms, but in the incompressible region, both acids exhibit the same extrapolated area at zero pressure. The authors suggested that the existence of the plateau would be interpreted as a transition state where the film-forming molecules change their conformation from a bent form, where the carboxylic and hydroxy groups are fixed to the aqueous phase, to an erect from, where the hydroxy group is forced from the subphase by compression. Thus, this transition is not due to a real condensation, as in the classical LE–LC transition.

On the other hand, Arnett and Gold [6], using impressing experimental conditions, reported attempts to detect an eventual enantiomer discrimination between pure D- and L-isomers of dipalmitoylphosphatidylcholine and their racemic mixture by ultra-high-field NMR spectroscopy, differential scanning calorimetry and monolayer techniques. They came to the conclusion that they were unable to demonstrate a significant difference between racemic DPPC and its enantiomers, and suggested that the absence of chiral discrimination is probably due to the inaccessibility of the chiral center. According to these data, it seems clear that chiral discrimination requires the involvement of the chiral sites in the interactions.

Conclusions

In the present work, attention was focused on the chiral discrimination occurring in *N*-acylamino acid monolayers. It has been shown that the racemic material gives a more expanded monolayer than that of the pure chiral compound. Through the phase diagram analysis of L-MA/D-MA binary mixtures, it was shown that the two antipodes are miscible in the expanded as well as in the condensed states, and the homochiral interactions are stronger than the heterochiral ones.

On the other hand, we failed to demonstrate any diastereoisomer discrimination in L-DMPC/L- or D-MA mixed monolayers, yet such discrimination is obvious when considering interfacial processes, such as hydrolysis of phospholipids by phospholipase A_2 or chemical communication between cells through interactions between chiral hormones and chiral cell membranes.

The reported results show that the monolayer technique provides a powerful approach to the understanding and control of stereospecificity. The possible application of this technique is far from being largely exploited in the field of the stereospecific phenomena, in spite of the publication of the authoritative review of Stewart and Arnett [21] dealing with chiral monolayers.

Acknowledgments

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