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MODEL MEMBRANE STUDIES OF SPIN-LABEL PROBES

PART 1. MIXED MONOLAYERS OF 12-NITROXIDE STEARIC ACID AND MYRISTIC ACID

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SUMMARY

Pure and mixed monomolecular films of a cell membrane spin label probe, 12-nitroxide stearic acid have been studied where myristic acid was selected as the host lipid. The behavior of 12-nitroxide stearic acid at the air water interface is understood in terms of two molecular configurations: erect (with only the carboxyl group in the interface) and bent (with both the carboxyl group and the oxazolidine ring in the interface). In mixed films both of these conformations play a role at high surface pressures. At low probe concentrations, 12-nitroxide stearic acid is primarily in an erect conformation, while at high probe concentrations the reverse is true. This particular host lipid appears capable of erecting the probe molecule with only small concentrations of myristic acid. In a condensed host lipid, the probe is partially immiscible, and segregates to form a heterogeneous film from which it is readily collapsed. The probe is seen to perturb the molecular packing in this mixed system and the perturbation to be dependent on both the molecular shape and nature of the probe.

INTRODUCTION

The selection of a mixed monomolecular film as a model membrane system for the study of the behavior of spin-labelled molecule has some obvious disadvantages as well as advantages. Thus, even though the energetics of a monolayer and bilayer are quite similar [1], they are not identical [2]. Perhaps more important, typical ESR spectra are obtained with a liposome or membrane host lipid : probe molar ratio of ≥ 100 : 1 while changes in the monolayer properties (surface pressure; surface potential; or surface shear viscosity [3]) are difficult to detect above concentrations of 50 : 1. On the positive side, however, this latter problem may be partially circumvented by assuming that behavior in the 40 to 30 : 1 range can be considered near ideal and extrapolated to the concentration which the probe "sees". In addition, the monolayer provides clear information concerning molecular packing and orientation, not only under membrane-like conditions, but also under more extreme situations.

The selection of myristic acid as the "host" lipid for the probe under consideration (12-nitroxide stearic acid) was based on several important considerations. At 21 °C the surface pressure-area/molecule isotherm is fully (liquid) expanded to approximately 17 dynes/cm and is not fully condensed until 20 dynes/cm. This means that the system can provide expanded and condensed physical states under membrane-like conditions. Previously we reported that 12-nitroxide stearic acid appeared partially immiscible in a condensed state [4], and it seemed worthwhile to confirm this situation. In addition behavior in mixed monolayers, in the absence of specific interactions, was thought to be primarily dictated by the physical state of the component amphipathic lipids and not by the precise molecular structure that leads to that state [5]. It, therefore, seemed wise to select a host lipid, as like the probe molecule as possible, which under experimental conditions, was primarily in the expanded state. Myristic acid seemed an obvious choice. In Part II of this series of papers we will show that specific interactions do, in fact, appear to occur between 12-nitroxide stearic acid and dipalmitoyl phosphatidylcholine and an understanding of the simple 12-nitroxide stearic acid-myristic acid system facilitates a better understanding of the probe-dipalmitoyl phosphatidylcholine specific interactions. Previously, we have presented data on this system at low probe concentrations [4, 6], we now report data for the complete system.

EXPERIMENTAL AND RESULTS

The 12-nitroxide stearic acid was a gift of Dr. J. D. Morrisett of the John Baylor College of Medicine, Houston, Texas. The sample was further purified by preparative thin-layer chromatography using an extra-pure silica gel, until a single spot was obtained under all developing conditions. The myristic acid, nominally 99+% pure, was purchased from Applied Science and was used as received. The water used as a substrate was doubly distilled from glass, the first time from alkaline permanganate. It was then distilled two more times from quartz. Surface areas were usually reproducible from film to film within ±0.5 $Å^2$/molecule, except for the pure 12-nitroxide stearc acid at very low surface pressures where the variation was somewhat greater. Surface potentials were reproducible within ±10 mV. The corresponding values for compression of a single film were 0.1 $Å^2$/molecule and 1 mV respectively. The isotherms (surface pressure vs. area/molecule) and corresponding surface potential plots are shown in Fig. 1.

Previously the isotherms, but not surface potential data, for low probe concentration films were published. In the preliminary report [6], some of the low probe concentration mixed film data were incorrectly evaluated resulting in slight changes in the isotherms (but significant changes in the mean molecular area plots). For this reason we present a complete set of data here for the entire compositional range. The isotherm data are represented in Fig. 3 as mean molecular areas as a function of film composition. The surface potentials are also presented as surface dipoles ($\mu_\perp$) in Fig. 2, thus eliminating the effect of changing area/molecule.

The myristic acid isotherm in Fig. 1 shows not only the usual liquid expanded-liquid condensed phase change at approximately 17 dynes/cm but also a second,

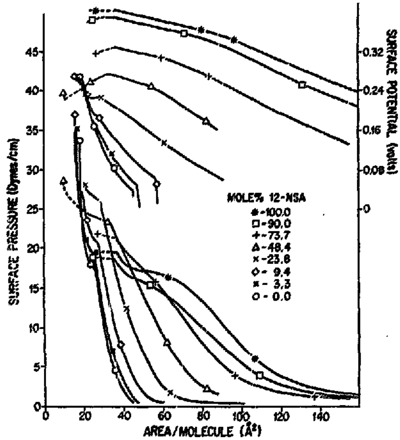

Fig. 1. Film characteristics of the myristic acid-12-nitroxide stearic acid at 20.8 °C. The upper set of curves are the surface potentials as a function of area/molecule. The lower set illustrates the surface pressure also as a function of the area/molecule. The curves were continuously recorded, the symbols are used only to designate the film composition. The broken lines indicate metastable regions or film collapse. 12-NSA, 12-nitroxide stearic acid.

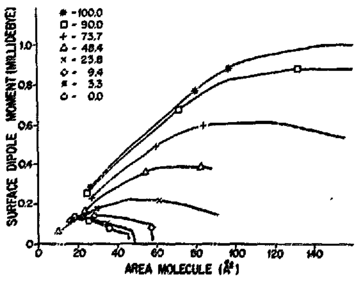

Fig. 2. Surface dipole (moments ($\mu_{\perp}$) as a function of area/molecule for the myristic acid-12-nitroxide stearic acid mixed film system at 20.8 °C. The curves were continuously recorded, symbols are used only to designate film composition expressed as mol % 12-nitroxide stearic acid. Broken lines indicate metastable regions or film collapse. 12-NSA, 12-nitroxide stearic acid.

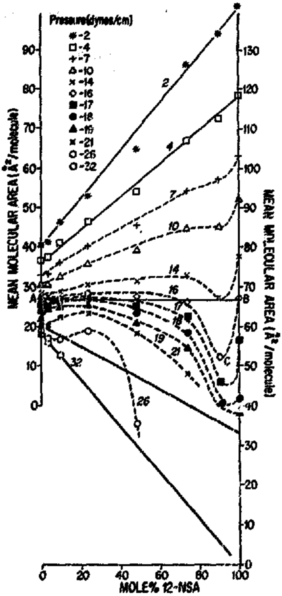

Fig. 3. Mean molecular areas as a function of film composition and surface pressure for the system myristic acid-12-nitroxide stearic acid at 20.8 °C. Solid lines indicate behavior for ideal mixing or nonmiscibility. Broken lines indicate actual behavior. Surface pressure are indicated both by the symbols indicated and by the number located close to each plot. The right hand ordinate represents area/molecule in Å² (displaced from left hand ordinate). Abscissa, film composition expressed as mol % 12-nitroxide stearic acid. 12-NSA, 12-nitroxide stearic acid.

though less evident, phase change at low areas/molecule reflecting a change in the close-packing of the alkane chains. As the probe concentration of the film is increased, the film undergoes an expansion and phase change shift, which is similar, but not identical, to that produced by raising the temperature [7]. The resemblance between the probe impurity effect and that of temperature is most evident at low probe concentrations (approx. 3 mol % 12-nitroxide stearic acid) but even here, differences are evident. Thus, at this concentration, a significant increase in the film collapse pressure is detectable and the collapse pressure appears to reach a maximum at about 9 mol % 12-nitroxide stearic acid. In contrast, the collapse pressure decreases slightly with increasing temperature. It should be noted that the collapse pressure variation indicates that the two components are at least partially miscible in the condensed state.

At concentrations between 9 and 50 mol %, the film isotherm dramatically changes from that of myristic acid. Thus at a 24 mol % concentration the collapse pressure has shifted to low pressures well below that of the pure myristic acid effectively eliminating the condensed portion of the isotherm. Moreover, the physical state of the film is difficult to define in that all films with lower probe concentrations are clearly liquid expanded at lower pressures and all films with higher probe concentrations are gaseous expanded. The 24 mol % film falls into an intermediate category, though there are indications of "lift-off" [3] from both surface pressure and potential data, suggesting that the film is probably best designated as liquid expanded.

By the time the molecular composition of the film attains a 50 mol % composition, its behavior is clearly dominated by the probe molecule. The film is gaseous expanded, it shows a barely detectable inflection (at about 17 dynes/cm) and has a near liquid collapse phase: all features of 12-nitroxide stearic acid at about 21 °C. It would appear that during collapse some film loss and segregation occurs as indicated by the sudden change in compressibility at 14 Å^2/molecule deposited. As the film concentration is changed to that of the pure probe molecule, the collapse pressure is lowered, the inflection becomes more pronounced and the film expansion at low pressures is increased.

The surface potentials (Fig. 1) show a similar pattern to the isotherms. The vertical region of the surface potential for the pure myristic acid film (as well as for the 3.3 and 9.4 mol % 12-nitroxide stearic acid films) represents the 2-phase liquid expanded-gaseous region. The subsequent break which appears for these low probe concentration films, reflects the liquid expanded-liquid condensed phase change. For the 23.8 mol % film, however, compression beyond this transition results in a slight negative slope suggesting that some film loss may be taking place and that this region has begun to resemble the collapse region for 12-nitroxide stearic acid. The 48.4 mol % film, having an almost 1 : 1 molar ratio composition is clearly gaseous expanded, shows an inflection and has a distinctly negative slope beyond the break until about 14 Å^2/molecule when a small vertical region is seen. It is interesting to observe that, as the composition is changed to that of pure 12-nitroxide stearic acid, this metastable collapse region assumes a decreasingly negative slope, until for 12-nitroxide stearic acid it is almost horizontal. This slope change indicates a shift from a solid to a more liquid-like collapse phase [3] with increasing 12-nitroxide stearic acid film content. The small vertical region for the 48.4 % film at 14 Å^2/molecule suggests that during collapse 12-nitroxide stearic acid is preferentially lost, resulting in a higher myristic acid content (and enhanced stability) for the last vestage of film to be collapsed. The collapse phases of the 73.7, 90.0 and 100 % 12-nitroxide stearic acid films could similarly be taken to lower areas/molecule, however, compression was stopped at the points indicated. The surface potential data are also represented in Fig. 2 as a function of the vertical component of the surface dipoles [3] $\mu_\perp$: where $\mu_\perp = A \cdot \Delta V/12\pi$ is the dipole moment in millidebye, ΔV is the surface potential in millivolts and A the area in Å^2/molecule. For 12-nitroxide stearic acid the inflection in the isotherm occurs at approximately 70 Å^2/molecule, but in the surface potential plot the primary change in slope occurs at 90 Å^2/molecule (Fig. 1). In the surface dipole plot (Fig. 2) a near linear rate of decrease is attained at 90 Å^2/molecule and remains constant until collapse occurs. The decrease in the surface dipole moment is continuous and shows no dramatic shift. As the 12-nitroxide stearic acid content of the mixed films decreases, a maximum appears in the $\mu_\perp$ plot (73.7 %). Ultimately the $\mu_\perp$ plot exhibits a more normal increase with decreasing area/molecule over almost the entire compressional range.

The isotherms are also represented in Fig. 3 as mean molecular area vs. composition plots. At low pressures the plots deviate little from linearity, however at pressures as low as 7 dynes/cm an interesting negative deviation from linearity occurs at high probe concentrations. This trend coupled with slight positive deviations (at low probe concentrations) continues and increases up to approximately 16 dynes/cm. Beyond this point the positive deviations (low probe concentrations) increase, while the negative deviations (high probe concentrations) decrease. Above 18 dynes/cm, pure 12-nitroxide stearic acid has collapsed and the compositional range of the mean molecular area plot is diminished. As the surface pressure examined is raised, the compositional range continues to diminish, until at 30 dynes/cm it is limited to probe concentrations below9 %.

DISCUSSION

In order to understand the behavior of 12-nitroxide stearic acid in mixed films it is essential to understand the film behavior of pure 12-nitroxide stearic acid. We have previously explained the isotherm in terms of a gaseous film at high areas/molecule and low pressures with both polar groups in the air-water interface. Compression results in the molecule assuming a bent conformation and eventually the forcing of the weaker polar group (the oxazolidine ring) out of the interface. This latter event was cited as an explanation of the inflection which occurs at approximately 70 Å^2/molecule [4]. Finally, the short condensed region was taken to represent a close-packed erect film of 12-nitroxide stearic acid molecules.

Comparison of the isotherm of 12-nitroxide stearic acid with that obtained for β-estradiol diacetate (also a bipolar amphipathic molecule) reveals that the latter molecule exhibits a sharp break rather than an inflection, when the molecule is forced from a horizontal conformation (with both polar groups in the interface) to a vertical conformation (with only one) [8]. The surface potential data for β-estradiol diacetate also show a sharp break and surface dipole data a 50 % decrease on removal of one of the polar groups [8]. For 12-nitroxide stearic acid the corresponding changes are a slight change in slope in the surface potential (Fig. 1) and a steady decrease in the surface dipole (Fig. 2). The data would seem to support a gradual change from a bent to an erect conformation for 12-nitroxide stearic acid.

Further evidence supporting this conclusion has been presented elsewhere in the extraordinary temperature dependence of films of 12-nitroxide stearic acid where, over at least a 40 °C temperature range, the films condense with increasing temperature [9]. In order to explain such behavior it is necessary to postulate that the bent and erect conformations can jointly coexist over a wide compressional range, though obviously the bent conformation predominates at high area/molecule and the erect conformation at low areas/molecule.

We now turn to considerations of the mixed films and, in particular, to the interpretation of the mean molecular area data illustrated in Fig. 3. When such data show a linear relationship at all concentrations, i.e.

$$A_m = \chi_1 A_1 + \chi_2 A_2$$

where A_m = molecular area for a mixed monolayer, A_1, A_2 = Areas occupied/molecule of pure component 1 and 2 respectively; and χ_1, χ_2 = mol fractions of components 1 and 2, we may assume either ideal or nonmixing of the two components. Deviations from this linear relationship indicate non-ideal behavior and miscibility. It should also be noted that such plots display the accommodation (positive or negative) of the probe by the system following probe insertion.

At low pressures the mean molecular area plots show essentially straight line relationships which at these low pressures (< 4 dynes/cm) and high areas (> 100 Å^2/molecule) are best taken as indicating near ideal mixing behavior. As the pressure is raised, however, a negative deviation appears at high (approx. 90 mol % 12-nitroxide stearic acid) probe concentrations for surface pressures ranging from approximately 7–17 dynes/cm. Comparison with the original isotherms in Fig. 1 reveals an excellent correspondence between the negative deviations of Fig. 3 and the existence of a bent conformation for 12-nitroxide stearic acid. Thus, the negative deviations reach a maximum at 16 dynes/cm when the pure 12-nitroxide stearic acid isotherm is passing through the inflection, but cease when the condensed region of the isotherm is reached at approximately 19 dynes/cm. On this basis we interpret the negative deviations at high probe concentrations as arising through the erection of 12-nitroxide stearic acid from the bent to the erect conformation. The fact that only small amounts of myristic acid are required to bring about this erection suggests that at low probe concentrations 12-nitroxide stearic acid is almost entirely in the erect conformation in this particular system.

The model membrane system becomes particularly interesting as relevant concentrations and surface pressures approach those thought to exist under membrane like conditions [1]. At 16 dynes/cm (Fig. 3) both the actual (broken line) and the ideal (solid line) behavior are depicted. Small positive deviations are evident over most of the concentration range with large negative deviations at high probe concentrations. These positive deviations are perhaps better understood if we assume that at point C in Fig. 3 the erection of 12-nitroxide stearic acid is complete. A line joining AC can be approximated as representing ideal behavior in a mixed monomolecular film where the two components are myristic acid and erect 12-nitroxide stearic acid. When this is done, we can see that the positive deviations from ideality become quite large. The point becomes clearer as we proceed to higher pressures. Thus at 17 and 18 dynes/cm the positive deviations become more pronounced and the negative deviations disappear as the pure 12-nitroxide stearic acid isotherm attains its short condensed region.

At low probe concentrations, the mixed film behavior is dominated by that of myristic acid and, between 18 and 19 dynes/cm, the delay of its phase change produces a small sharp change in slope in the mean molecular area plot. Above this pressure the increase disappears and the data indicate a shift to either ideality or non-miscibility with increasing surface pressure (21–32 dynes/cm). This observation confirms our previous conclusion that when the host lipid is in a condensed state, most of the probe molecules will be squeezed out.

Above approximately 19 dynes/cm, we no longer have data for pure 12-nitroxide stearic acid and, therefore have difficulty in placing the line representing ideal behavior. A reasonable procedure would be to extrapolate the condensed region of the pure 12-nitroxide stearic acid isotherm to higher pressures to obtain the area/molecule in mixed films (i.e., to assume that the compressibility of 12-nitroxide stearic acid remains essentially at the value obtained in the 18–19 dynes/cm region). This gives us theoretical values of between 38 and 28 Å^2/12-nitroxide stearic acid molecule as the surface pressure is raised from 21 to 32 dynes/cm. Indeed, the data at 21 dynes/cm do appear to approach a limit of approximately 33 Å^2/12-nitroxide stearic acid molecule. However, the data at 26 dynes/cm, particularly the point nominally at 48.4 mol % 12-nitroxide stearic acid, would fall below a line drawn to 28 Å^2/12-nitroxide stearic acid molecule and would seem to better approximate to the zero area value for 12-nitroxide stearic acid. All the 32 dynes/cm data (three points) clearly extrapolate to a zero area value for 12-nitroxide stearic acid. We, therefore, conclude that significant amounts of 12-nitroxide stearic acid are lost from the film on compression at 26 dynes/cm and that the bulk of the 12-nitroxide stearic acid is lost at 32 dynes/cm. The isotherm shapes at these pressures (Fig. 1) indicate that the most likely mechanism for this loss is lateral diffusion followed by collapse of the 12-nitroxide stearic acid from the 12-nitroxide stearic acid rich phase.

Biological implications

Using the study of a monolayer model membrane system, we have been able to demonstrate three interesting aspects of the use of a cell membrane spin-label probe (12-nitroxide stearic acid). First of all, the probe clearly perturbs its own environment as indicated by the host lipid phase-change shift. The magnitude of this perturbation may be approximated by comparing the effects of increasing temperature [7] and increasing probe content (Fig. 1). The procedure we have adopted has been to observe the perturbation at low probe concentrations where most probe molecules are surrounded by host lipids (approx. 3 mol %, 12-nitroxide stearic acid) and to extrapolate that effect to the concentration the probe "sees". One approximation to this concentration would be to consider only nearest neighbours or a molar ratio of host lipid to probe of 6 : 1. It is clear however that such probes can detect cooperative motion in lipids and hence can "see" beyond nearest neighbour interactions. Kleeman and McConnell have discussed such regions of decreasing perturbation in the formation of "halos" [10]. Unfortunately, the precise way in which this effect falls off is not known though similar calculations [11] suggest that it will be rapid and that an equivalent molar ratio of between 12 : 1 and 15 : 1 might be realistic. On this basis we find for this system a net perturbation equivalent to a temperature increase of 2–4 °C.

The positive deviations in the mean molecular area plots, particularly evident when considering an erect 12-nitroxide stearic acid molecule, indicate that there is a negative accommodation of the probe in the system following its insertion. Thus after the initial perturbation due to a differing shape of the probe molecule, there is a further perturbation presumably due to its polar nature [12].

The second interesting aspect is the ability of 12-nitroxide stearic acid to adopt a bent conformation at the air-water interface. In mixed films only small amounts of myristic acid are required to erect the probe molecule. It would seem likely that under membrane like conditions only a small fraction of the 12-nitroxide stearic acid could be in the bent conformation. However, it should be made quite clear that this observation is made for this particular probe and this particular host lipid. Data to be presented in a successive paper [13] will make it clear, that while such a conformation probably still plays only a minor role, with 12-nitroxide stearic acid in a lecithin, it should no longer be considered negligible. It would be reasonable to suggest that the presence of a bent conformation for 12-nitroxide stearic acid would give rise, in part, to the "liquid line" seen in many ESR spectra [14]. This cannot however be established since Butler et al. [15], have convincingly demonstrated that the primary cause of such liquid lines are freely rotating probe molecules in solution. It should also be noted that the stability of such a bent conformation is clearly a function of the location of the oxazolidine ring on the hydrocarbon chain [16].

Finally, we have once again observed evidence which indicates partial immiscibility of this probe in a lipid condensed state and it would seem that when the host lipid is fully condensed only a few probe molecules can remain. This corresponds well with the report of Hubbell and McConnell [17] that probe concentrations of 12-nitroxide stearic acid in condensed bilayers cannot exceed a molar ratio (host : probe) of 100 : 1.

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REFERENCES

1 Phillips, M. C. and Chapman, D. (1968) Biochim. Biophys. Acta 163, 301–313
2 Danielli, J. F. (1968) in Molecular Associations in Biology, pp. 529–537, Academic Press, New York
3 Cadenhead, D. A. (1969) Ind. Eng. Chem. 61, 22–28
4 Cadenhead, D. A. and Müller-Landau, F. (1973) Biochim. Biophys. Acta 307, 279–286
5 Cadenhead, D. A. (1970) in Recent Progress in Surface Science, Vol. 3, pp. 169–192, Academic Press, New York
6 Cadenhead, D. A. and Müller-Landau, F., (1973) in Proc. XXIst Coll. Protides of the Biological Fluids (H. Peeters ed.) pp. 175–182, Pergamon Press, London
7 Adam, N. K. and Jessop, G. (1927) Proc. Roy. Soc. Lond. A112, 376
8 Cadenhead, D. A. and Phillips, M. C. (1967) J. Colloid Interface Chem. 24, 491–499
9 Cadenhead, D. A. and Müller-Landau, F. (1974) J. Colloid Interface Sci. 49, 131–134
10 Kleeman, W. and McConnell, H. M. (1976) Biochim. Biophys. Acta 419, 206–222
11 Phillips, M. C., Cadenhead, D. A., Good, R. G. and King, H. F. (1971) J. Colloid Interface Sci. 37, 437–442
12 Cadenhead, D. A., Kellner, B. M. J. and Müller-Landau, F. (1975) Biochim. Biophys. Acta 382, 253–259
13 Cadenhead, D. A. and Müller-Landau, F., in the press
14 Landsberger, F. R., Lenard, J., Paxton, J. and Compans, R. W. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2579–2583
15 Butler, K. W., Tattrie, N. H. and Smith, I. C. P. (1974) Biochim. Biophys. Acta 363, 351–360
16 Cadenhead, D. A. and Müller-Landau, F. (1975) Adv. Chem. Ser. 144, 294–307
17 Hubbell, W. L. and McConnell, H. M. (1971) J. Am. Chem. Soc. 93, 314–326