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PURE AND MIXED MONOMOLECULAR FILMS OF 12-NITROXIDE STEARATE

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SUMMARY

Pure and mixed monomolecular films of 12-nitroxide stearic acid (12-nitroxide stearate) have been studied at the air–water interface and both surface pressures and surface potentials obtained as a function of area/molecule. The isotherm for the pure compound shows an inflection at approx. 70 $Å^2$/molecule and a collapse at 39 $Å^2$/molecule. These observations indicate that close-packed films occur with both polar groups (carboxyl and nitroxide) and then with one polar group (carboxyl) located at the air–water interface. The pure film shows a slow deterioration with time at both pH 2 and 6 due to an attack on the nitroxide group.

In mixed films positive deviations from ideality occur at higher pressures with a trend toward small negative deviations at low pressures. Under membrane-like conditions the free energy of chain–chain interactions is reduced. The probe proved to be partially immiscible in a liquid condensed film and immiscible in a solid condensed film, an observation in accordance with a similar segregation observed below the liquid crystalline–gel transition in liposomes and membranes.

INTRODUCTION

In order to investigate possible changes in both packing and molecular interactions arising through the introduction of a spin label on a membrane lipid, we have carried out studies of both pure and mixed monomolecular films of such probes at the air–water interface[1,2]. These previous studies involved two rigid amphipathic molecules: 3-nitroxide androstane and 3-nitroxide cholestane. Unfortunately the 3-nitroxide androstane proved too unstable for meaningful film work, but the studies of 3-nitroxide cholestane showed that in many ways this molecule can act as a substitute for cholesterol and as such may be considered an acceptable membrane lipid. More recently Tinoco *et al.*[3] also reported work with 3-nitroxide cholestane. Their findings essentially agreed with ours though there were some discrepancies.

Tinoco *et al.*[3] also reported for the first time an isotherm for 12-nitroxide stearate. We believe that our results significantly extend their findings for pure films of this spin-label probe and in addition we report the behavior of selected mixed films; behavior which is presumably of direct bearing on the situation in both liposomes and membranes. The behavior of this probe is of special interest since,

unlike those selected in our previous work[1,2], this molecule has a flexible acyl chain to which the nitroxide group is added. Previous work with branched chain surfactants, suggests that, at the very least, the added nitroxide group must affect chain packing of nearest neighbor molecules[4].

EXPERIMENTAL AND RESULTS

A sample of 12-nitroxide stearate was generously provided by Dr J. D. Morrisett of the Baylor College of Medicine, Houston, Texas. Initially, the sample was purified using preparative thin-layer chromatography, with a Merck (Darmstadt) silica gel PF-254, selected in part because of the absence of a binder. Relatively large thick plates were used to ensure an adequate amount of sample. Approx. 100 mg samples of the initial impure material were dissolved in ethylene chloride and applied to the plate which was then developed in ether–hexane (3:7, v/v). On separation the ultraviolet active band was removed, washed in ether–hexane and the residue filtered, after which the solvent was evaporated under vacuum. The probe sample was then redissolved in methylene chloride in order to remove any remnants of silica gel. After reevaporation, the final yield of solid was 38.5 mg. This material was found to melt between 9.5 and 10 °C using a temperature program of 0.5 °C/min. The other film materials, stearic and myristic acids, were purchased from Applied Science Laboratories as 99.5% pure and were used as received.

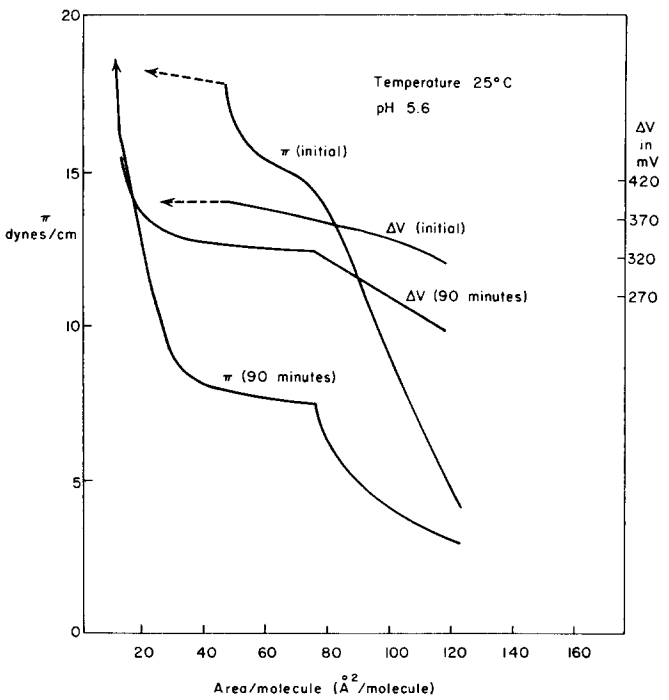


Fig. 1. Monomolecular films of 12-nitroxide stearate, temp. 25 °C, pH 5.6. Left-hand ordinate: surface pressure in dynes/cm. Right-hand ordinate: surface potential in mV. Abscissa: area/molecule in Å²/molecule.

The film balance and general techniques[5] as well as modifications of these techniques for mixed films[6] have been adequately described elsewhere. The particular films examined in this study showed high reproducibility and reversibility (generally within a 1% error). Because of this and because of the continuous method of film compression, it proved possible to follow gradual changes in the isotherms as a function of time. A qualitative check was made of the ESR signal after spreading a pure film for 15 min and this proved positive.

The results are shown in Figs 1–4. Figs 1 and 2 show isotherms and surface potentials of 12-nitroxide stearate as a function of time at a pH of 5.6 and 2, respectively. A remarkable feature of this particular film was its reversible behavior, even after film collapse, a feature which permitted the repeated observation of the total isotherm as a function of time. Only two isotherms and the corresponding surface potential curves are shown (the initial and one subsequent isotherm) at each pH, however, numerous other isotherms determined at regular time intervals showed a systematic change as a function of time.

Figs 3 and 4 illustrate the behavior for mixed films with stearic and myristic

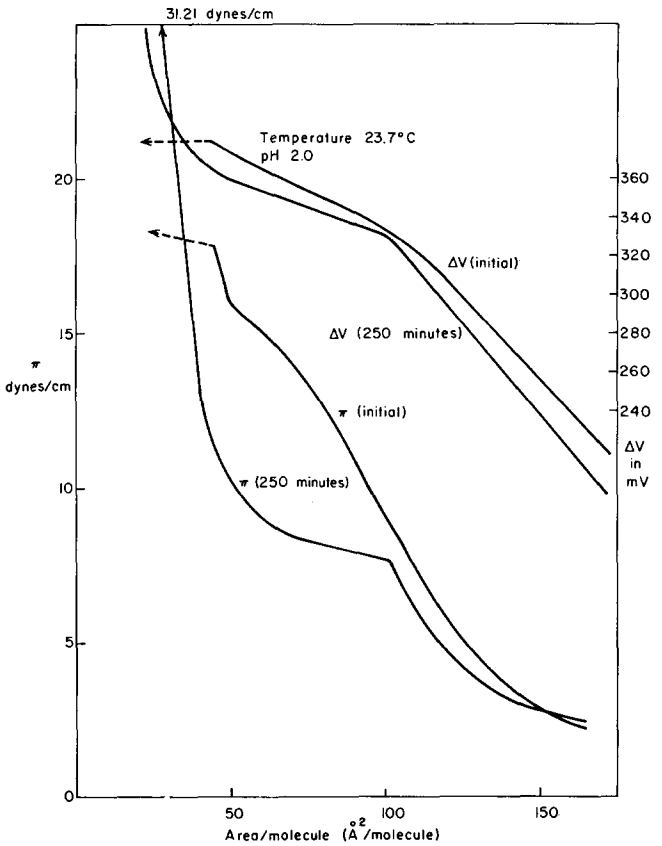
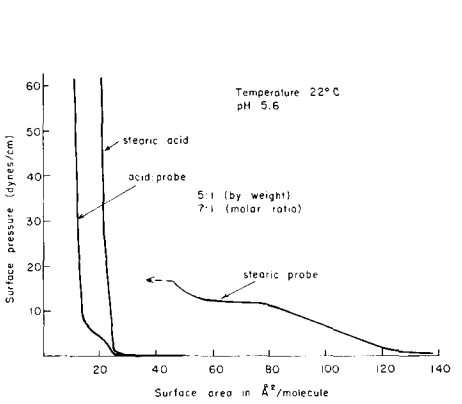


Fig. 2. Monomolecular films of 12-nitroxide stearate, temp. 23.7 °C, pH 2. Left-hand ordinate: surface pressure in dynes/cm. Right-hand ordinate: surface potential in mV. Abscissa: area/molecule in Å²/molecule.



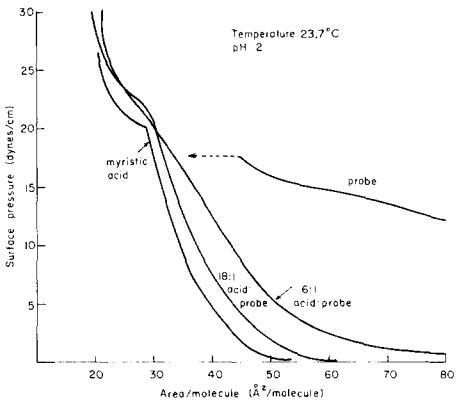


Fig. 3. Monolayers of stearic acid, 12-nitroxide stearate and a 7:1 (molar ratio) mixed film at 22 °C and pH 5.6. The mixed acid–probe isotherm was obtained on a second compression. Ordinate: surface pressure in dynes/cm. Abscissa: surface area in Å²/molecule.

Fig. 4. Monolayers of myristic acid, 12-nitroxide stearate and a 6:1 also a 18:1 (molar ratio) mixed film at 23.7 °C and pH 23. Ordinate: surface pressure in dynes/cm. Abscissa: surface area in Å²/molecule.

acids, respectively. For the mixed films the behavior was essentially the same irrespective of the pH of the substrate. The behavior shown is that of the initially spread film. A check was also made of the time dependence of the mixed myristic acid-probe films. No obvious time dependence was observed, however, if for this system the film was taken beyond the collapse point, subsequent isotherms were displaced to smaller areas/molecule. A point of interest was that while the collapse of the pure film was indicated by a slight positive gradient of the surface pressure–area (π–A plot, the initial mixed myristic films (all compositions) showed a sharp drop in surface pressure at areas/molecule beyond collapse.

DISCUSSION

Pure films

Examination of the isotherms of the 12-nitroxide stearate, at either pH 5.6 or 2 (Figs 1 and 2), reveals that the molecule exists in a much more expanded state than does stearic acid (see also Fig. 3). Furthermore, in the isotherm of the probe taken immediately after spreading, there is a distinct inflection at 70 Å²/molecule. The above observations can only be explained if it is assumed that the nitroxide probe is located at the air–water interface. A similar conclusion was reached by Tinoco *et al.*[3].

The experimental techniques employed by us, however, have revealed new aspects of the film behavior of this probe. Thus, since a typical isotherm compression took approx. 5 min, and since the film was essentially stable over such time periods[5] we were able to take a series of "instantaneous" pictures of the isotherm as a function of time. Initially the isotherm shows an inflection at about 70 Å²/molecule and collapse at 39 Å²/molecule, areas which show an excellent correspondence using Corey–Pauling–Koltun molecular models with close packed films where both can

boxyl and nitroxide groups (70 Å^2) and only the carboxyl group (39 Å^2) are located in the interface. At the inflection point (70 Å^2) the surface potential (ΔV) shows a steady change with change in area/molecule but the ΔV–A plot slope abruptly becomes zero at the collapse point.

All of the above indicate that in a pure film of 12-nitroxide stearic acid both polar groups are located at the interface in an expanded state and remain in this situation until a close packed film is achieved. Further compression results in the gradual removal of the nitroxide group (the least polar) from the surface, until finally a close packed film is achieved at 39 Å^2 where the probe is located in the hydrophobic portion of the film.

Examination of the film after remaining at the interface for a prolonged period of time at the air–water interface reveals that profound changes are taking place. Stearic acid itself shows no such changes, therefore, the effects must be associated with the nitroxide group. Since the condensed portion of well exposed films exhibit areas/molecule in the 10–20 Å^2/molecule region, we can only conclude that loss of material from the film must have taken place. Clearly, in some way, the nitroxide group is being attacked with the resultant product being lost from the film. That a partial ESR signal is observed after a period of 15 min at pH 2, indicates that such a process is taking place. Since the most likely change would be an acid hydrolysis of the probe, the apparent lack of a significant pH dependence is surprising. However, it should be borne in mind that the kinetics we observe are dependent on parameters not completely controlled (*e.g.* the number of film compressions within a given time) and may well have the solution of the reaction product in the substrate as the rate controlling step. The isotherm of Tinoco *et al.*[3] showing a pre-collapse area of 20 Å^2/molecule, may also be interpreted as indicating a similar loss of film material.

Mixed films

In Fig. 3 isotherms are shown for a mixed stearic acid-probe film (molar ratio 7:1) as are isotherms of pure stearic acid and the 12-nitroxide probe for comparative purposes. The choice of stearic acid as a mixed film component seemed an obvious one since it differs only in the substitution of two hydrogen atoms for the oxazolidine nitroxide group. Moreover, we have previously shown that the behavior of the mixed film will be primarily decided by the physical state of the films of pure components[6]. Stearic acid is liquid condensed at low pressures and solid condensed at higher pressures*. Thus, the results obtained for this system should be valid for other condensed gel or crystalline type films. What the results clearly show is that the two components are immiscible at all except possibly the lowest pressures (< 10 dynes/cm). There, in the liquid condensed region the probe is partially miscible. The low areas/molecule (< 20 Å^2/molecule) above about 10 dynes/cm can only be explained by film loss resulting in the use of too large a number of molecules in evaluating the abscissa in Fig. 3.

A contrasting situation is revealed in Fig. 4 which illustrates the isotherms for the probe, for myristic acid (liquid expanded below 20 dynes/cm and liquid condensed above) and for two mixed films (molar ratios acid to probe 18:1 and 6:1). For this system the two components are clearly miscible in the liquid expanded state

* For a description of such terms see ref. 7.

and partially miscible in the liquid condensed. Thus, the areas/molecule are realistic, the sharpness of the myristic acid liquid condensed–liquid expanded phase transition decreases with increasing probe concentration, the collapse phase has essentially the same composition as that of the film (as indicated by lateral isotherm displacement for residual film after a previous collapse), and finally the mean molecular areas deviate from additivity[6]. However, at higher pressures (20–30 dynes/cm) it is possible to detect a shift to lower areas/molecule in the mixed films, particularly the 6:1, acid–probe mixture, the isotherm for the latter crossing that of the 18:1 mixture at 20 dynes/cm. This observation, coupled with that of the decrease in sharpness of the phase transition, indicates only a partial miscibility in the liquid condensed state.

The way in which the areas/molecule deviate from ideality are particularly interesting. For the film having a molar composition of 18:1, positive deviations from ideality were observed above 15 dynes/cm. The same film behaved in an essentially ideal fashion from 15 to 10 dynes/cm, while below 10 dynes/cm slight negative deviations were found. For the 6:1 film, the transition from positive deviations to ideal behavior occurred about 13 dynes/cm and from ideality to negative deviations at 7 dynes/cm. A 2.5:1 film (not illustrated) showed positive deviations at all surface pressures, the magnitude of the deviation increasing with increasing pressure. The deviations are however small and are almost within experimental error.

Membrane study implications

The observation that the nitroxide group has a tendency to locate at the air-water interface is of considerable interest and immediately raises the question as to the likelihood of such a conformation in a liposome or membrane. In order to evaluate this properly it should be realized that typical monomolecular film spreading procedure involves initially spreading the film as a gaseous monolayer providing a maximum opportunity for all polar groups to locate at the interface. Such a situation is much less likely to occur in a liposome or a membrane, especially if we accept that the packing in a membrane is probably similar to that in a monolayer at a surface pressure of not less than 20 dynes/cm*.

Nevertheless, the possibility that a small fraction of the probe molecules may be folded such that both the nitroxide and polar group are in the polar region of the membrane lipids is a distinct possibility. Such as conformation could result in a more isotropic movement and provide an explanation for the "liquid line" observed in the ESR spectra of Landsberger *et al.*[10], using similar probes. The small negative deviations at low pressures observed for the 6:1 and 18:1 mixed films mean that when the probe molecule is surrounded by near vertically oriented film molecules there will probably be some tendency to erect the probe. Positive deviations which occur at all concentrations at higher surface pressures (> 15 dynes/cm) indicate that the free energy of chain–chain interactions is significantly affected (reduced) at relevant membrane pressures. At this time, we cannot yet resolve this deviation into enthalpy and entropy effects, however, it seems unlikely that our observations can be explained solely in terms of changes in packing (entropy) without at least some change in chain interactions. The dramatic lowering of the melting point (about 60 °C

* An evaluation based on a comparison of membrane transitions[8] and monolayer transitions[9]

between stearic acid and 12-nitroxide stearate further supports this point of view. It seems likely that positive deviations (reduced free energies of chain–chain interactions) will predominate over negative interactions (tendency to erect the probe molecule) under reasonable surface pressure conditions and membrane concentrations. We are, of course, faced with the problem that, as we proceed to concentrations where probe–probe interactions are effectively ruled out, a quantitative evaluation of any deviation from ideality becomes increasingly difficult. Nevertheless, because of the importance of these studies in understanding the behavior of spin-label probes in membranes, we are continuing to work on the quantitative aspects of such behavior.

An equally important observation is that as the environment of the probe changes from a liquid expanded to a liquid condensed or solid condensed state the probe begins to segregate and gradually squeeze out. Careful evaluation of our results suggests that this takes place by horizontal segregation to form patches of probe molecules which then collapse at essentially the collapse pressure of the pure probe film. The possibility, however, that some probe loss takes place directly in a perpendicular direction cannot be completely ruled out. If we accept that this transition is the equivalent of a liquid crystalline–gel transition[11], then we might expect that under similar circumstances spin-label probes would segregate. Thus, in a liposome or membrane on proceeding from above to below the liquid crystalline–gel transition temperature (T_c) we should observe some segregation of probe molecules. Recently, there have been several such reports[12–16]. McConnell *et al.*[13] have reported such a segregation as occurring at low temperatures in sarcoplasmic recticulum membranes (but not in the extracted lipid liposomes). They also interpret previously reported data[12] as indicating segregation of fatty acid probes in phosphatidylcholine liposomes at low temperatures when the probe/phosphatidylcholine ratio is $\geqslant 1:100$. Thus, the possibility of probe segregation is clearly dependent on the probe used and its concentration, as well as the precise lipid or membrane system. Sackmann and Träuble[14–16] have reported similar findings for mixed dipalmitoyl phosphatidylcholine–3-nitroxide androstane systems. The effect of cholesterol on such segregation is also of interest. In one instance (Jost, P. C., personal communication) multilamellar liposomes of dipalmitoyl phosphatidylcholine with 12-nitroxide stearate incorporated above the T_c, showed spin–spin interactions on cooling below this temperature and removing excess water. However, when the same probe was similarly incorporated into a 1:1 dipalmitoyl phosphatidylcholine–cholesterol mixture, spin–spin interactions were absent. The addition of cholesterol can prevent a true crystal lattice form forming and it would appear that this impurity effect[17] is sufficient to preserve the miscibility of the spin-label probe. These observations confirm that 12-nitroxide stearate will not remain fully miscible in a crystalline or gel-like state.

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REFERENCES

1 Cadenhead, D. A. and Katti, S. S. (1971) *Biochim. Biophys. Acta* 241, 709–712
2 Cadenhead, D. A., Demchak, R. J. and Müller-Landau, F. (1972) *Ann. N. Y. Acad. Sci.* 195 218–223
3 Tinoco, J., Ghosh, D. and Keith, A. D. (1972) *Biochim. Biophys. Acta* 274, 279–285
4 Izawa, M. (1952) *Bull. Chem. Soc. Jap.* 25, 182–187
5 Cadenhead, D. A. (1969) *Ind. Eng. Chem.* 61, 22–28
6 Cadenhead, D. A. and Phillips, M. C. (1968) *Adv. Chem. Ser.* 84, 131–148
7 Gaines, Jr, G. L. (1966) in *Insoluble Monolayers at the Air–Water Interface*, p. 156, Wiley Interscience, New York
8 McElhaney, R. N. and Tourtellotte, M. E. (1969) *Science* 164, 433–434
9 Cadenhead, D. A., Demchak, R. J. and Phillips, M. C. (1967) *Kolloid Z. Z. Polym.* 220, 59–6
10 Landsberger, F. R., Paxton, J. and Lenard, J. (1971) *Biochim. Biophys. Acta* 266, 1–6
11 Phillips, M. C. and Chapman, D. (1968) *Biochim. Biophys. Acta* 163, 301–313
12 Hubbell, W. L. and McConnell, H. M. (1971) *J. Am. Chem. Soc.* 93, 314–326
13 McConnell, H. M., Devaux, P. and Scandella, C. J. (1972) in *Membrane Research* (Fox, C. F ed.), pp. 27–37, N.Y.
14 Sackmann, E. and Träuble, H. (1972) *J. Am. Chem. Soc.* 94, 4482–4491
15 Sackmann, E. and Tràuble, H. (1972) *J. Am. Chem. Soc.* 94, 4492–4498
16 Träuble, H. and Sackmann, E. (1972) *J. Am. Chem. Soc.* 94, 4499–4510
17 Cadenhead, D. A. (1970) in *Recent Progress in Surface Science* (Danielli, J. F., Riddiford, A. C and Rosenberg, M. D., eds), Vol. 3, pp. 169–192, Academic Press, New York.