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A COMPARISON OF A SPIN-LABEL AND A FLUORESCENT CELL MEMBRANE PROBE USING PURE AND MIXED MONOMOLECULAR FILMS

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Summary

Monocular film studies of 12-nitroxide stearic acid and 12-(9-anthroyl) stearic acid reveal that deviations from the behavior of the parent molecule (stearic acid) are as much dictated by the polar, or nonpolar, nature of the probe group as by its size. In mixed films under membrane-like conditions, the spin label probe, 12- nitroxide stearic acid, exhibits positive deviations from ideality and should read too high a fluidity. The picture is, however, complicated by a tendency of this probe molecule to adopt a bent conformation, a tendency apparently enhanced by specific interactions with the lecithin zwitterion. 12- (9-anthroyl) stearic acid, in contrast, shows only negative deviations from ideality in mixed dipalmitoyl lecithin films and should read too low a fluidity.

In discussing the use of molecular probes in the analysis of membrane structure or fluidity, many reports point out that the probe can cause some perturbation of its own environment and modification of the quantitative results may be required. What is frequently not pointed out is that the degree of any such perturbation may vary considerably from one probe to the other. Thus, using monomolecular films as a model membrane system, we have demonstrated that, while 3-nitroxide cholestane [4′, 4′-dimethyl-spiro (5α-cholestane-3, 2-oxazolidine)-3′-yloxyl] behaved in a very similar way to cholesterol [1], 12-nitroxide stearic acid [2- (10-carboxydecyl) -2-hexyl-4, 4-dimethyl-3-oxazolidyloxyl] behaved in a totally different way from stearic acid, and under membrane-like conditions appeared to "see" an environment approx 10°C

higher than the untagged molecule [2]. We have extended such studies here to a comparison of a spin-label probe [3] [12-nitroxide stearic acid] and a fluorescent probe [4] [12- (9-anthroyl) stearic acid]. Both probes are formed by substitution of the "parent" molecule (stearic acid) at the 12-position. The

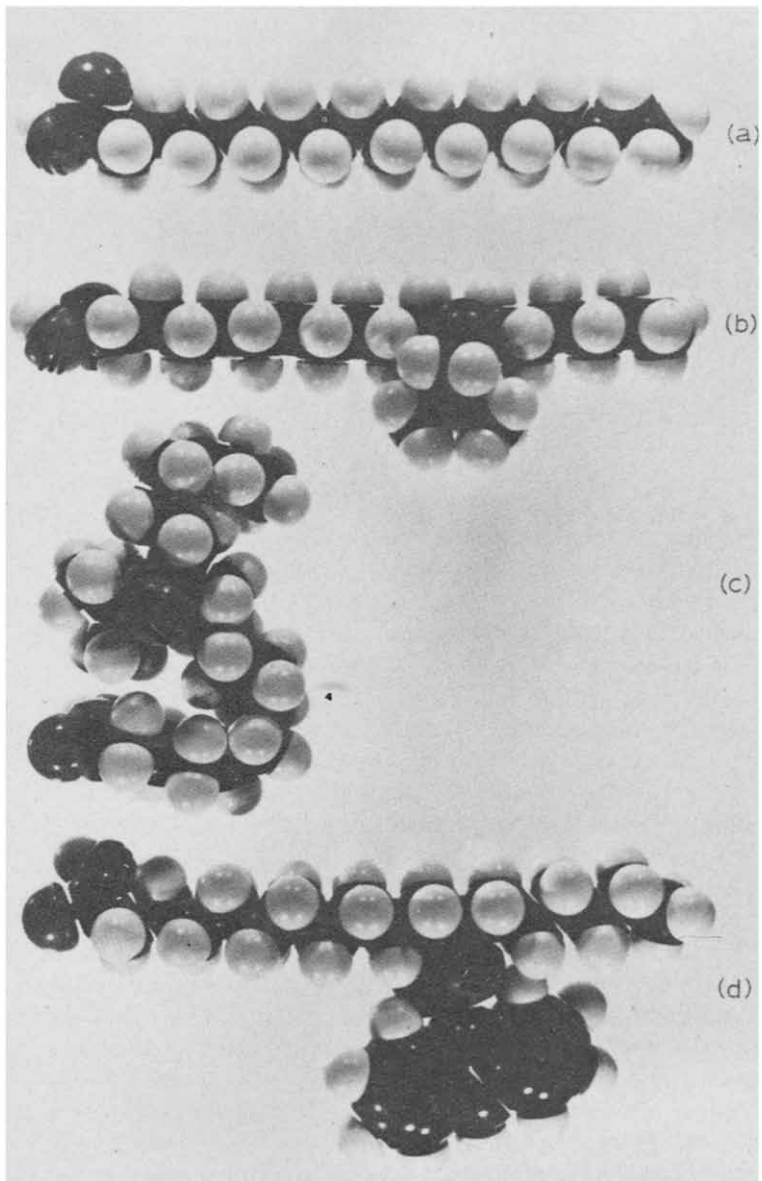

Fig.1. C.P.K. molecular models of (a) stearic acid; (b) 12-nitroxide stearic acid in the erect conformation; (c) 12-nitroxide stearic acid in the bent conformation with both polar groups in the interface; (d) 12-(9-anthroyl) stearic acid.

structure of both probes and that of stearic acid are shown in Fig.1. Examination of Fig.1 would suggest that the introduction of the much larger anthroyl group would cause a greater perturbation than would the oxazolidine (nitroxide) ring. As we will demonstrate, however, even a preliminary monomolecular film comparison reveals a much different picture and brings out some interesting features concerning the use of such probes in evaluating cell membrane properties.

The data reported here were obtained on an automated Wilhelmy plate film balance capable of recording both surface pressure and surface potential as a function of area/film molecule. A description of the apparatus and experimental techniques has been published elsewhere [5]. The 12-nitroxide stearic acid was a gift from Dr J.D. Morrisett of the Baylor College of Medicine, Houston, Texas, while the 12- (9-anthroyl) stearic acid was a gift from Dr R.A. Badley of Unilever Research, The Frythe, Welwyn, England. Both compounds were purified using preparative thin-layer chromatography with ultra-pure silica gel until a single spot was observed with at least two developing fluids. The 12-nitroxide stearic acid melted at 13° C [2], while the 12- (9-anthroyl) stearic acid melted at 79° C in accordance with the literature [4]. The stearic acid was obtained from Applied Science Laboratories and had a nominal purity of better than 99%. The sample used melted at 70° C. Except for 12-nitroxide stearic acid at high areas/molecule, areas/molecule were established within ± 0.5Å^2 for a given surface pressure. Surface potentials were reproducible within ±10mV for successive films but changes of 1mV could readily be detected during the compression of any single film. Water used for the film substrates was twice distilled from glass (the first time from alkaline permanganate) and then subsequently twice distilled from quartz.

(a) Pure Films

The results obtained for pure-films are illustrated in Fig.2. The stearic acid isotherm and surface potential data are in close agreement with other data pubblished in the literature [6]. A liquid condensed - solid condensed phase change is detectable at about 18 dynes/cm. At all pressures the stearic acid is in a condensed state. In contrast 12-nitroxide stearic acid is highly (gaseous) expanded. Clearly, the substitution of the oxazolidine ring for two hydrogen atoms at the 12-position of the stearic acid chain has had a dramatic effect on its film behavior. We have previously explained this effect by taking into account the polarity of the oxazolidine ring and regarding 12-nitroxide stearic acid as a bipolar amphipathic molecule [2, 7, 8, 9]. At high areas/molecule and low surface pressures the molecule is envisaged as lying in a near parallel orientation to the interface with both polar groups (the carboxyl and the oxazolidine ring) located in the interface. As the film is compressed the molecule assumes a bent conformation (Fig.1(c)) with the oxazolidine ring (the weaker polar group) still spending most of its time in the interface. Beyond the isotherm inflection (approx. 70 Å^2/molecule), however, the bent conformation becomes unstable and all oxazolidine rings are forced out of the water with only an erect conformation (Fig.1(b)) remaining stable in the small condensed region (approx.40 Å^2/mol-

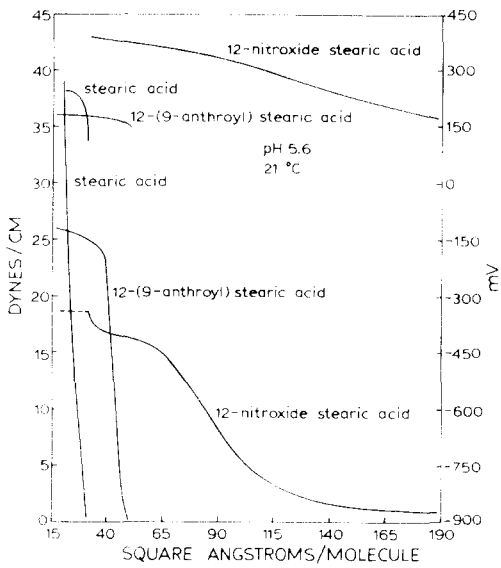

Fig. 2. Force-Area plots of stearic acid, 12-(9-anthroyl) stearic acid and 12-nitroxide stearic acid. Corresponding surface potential-area plots for the same compounds (as indicated by initials) are shown in upper portion of the figure. Left hand ordinate: Surface pressure in dynes/cm. Right hand ordinate: Surface potential in millivolts.

ecule). The concept of a dynamic conformational equilibrium for films $\geqslant 70$ Å^2 /molecule is necessary to explain the extraordinary temperature dependence of this [8] and other [9] nitroxide stearic acid films where condensation with increasing temperature is observed over at least a 40° C range (10—50° C). Interestingly enough, the conformational equilibrium is strongly dependent on the precise point of attachment of the oxazolidine ring on the stearic acid carbon chain [9]. Finally, the near horizontal broken line symbolizing the collapse phase indicates the liquid-like nature of this material [5].

12-(9-anthroyl) stearic acid unlike 12-nitroxide stearic acid is (liquid) condensed at all surface pressures. In spite of the ester portion of the anthroyl group the additive is essentially hydrophobic and the molecule behaves as a monopolar amphipathic molecule. From Fig. (d) we see that, with the anthracene aligned parallel to the alkane chain, a close-packed area/molecule should be approx. 40 Å^2. Collapse for this film is gradual and appears to commence around 20 dynes/cm. Like stearic acid, but unlike 12-nitroxide stearic acid, 12-(9-anthroyl) stearic acid does not respread easily from the collapsed material. Again, unlike 12-nitroxide stearic acid, 12-(9-anthroyl) stearic acid in a pure film, appears to exist only in an erect conformation.

The surface potentials for stearic acid and 12-(9-anthroyl) stearic acid are essentially the same. Thus, the anthroyl group has little or no effect on the surface potential. While this is not surprising since in 12-(9-anthroyl) stearic acid the point of attachment is well removed from the carboxyl or the terminal methyl group, it also confirms the conclusion drawn above, that the anthroyl

group does not get into the air-water interface. The surface potentials for 12-nitroxide stearic acid are also interesting in that while an inflection (corresponding to that in the isotherm) is apparent at approx. 70 $Å^2$/molecule, the change in slope is very slight. A plot (not shown) of the vertical component of the surface dipole ($\mu_\perp$) [5] shows a slight maximum at approx. 70 $Å^2$/molecule. This behavior should be contrasted with other bipolar amphipathic molecules where one polar group is being forced out of the interface. Thus β-estradiol diacetate shows a dramatic drop in $\mu_\perp$ when the molecule switches from an all-horizontal to an all-vertical orientation[10]. Clearly, the change in 12-nitroxide stearic acid is much more gradual with the molecule changing from a primarily bent conformation at areas/molecule > 70 $Å^2$, to a primarily erect conformation at areas/molecule less than this.

One may summarize the information on the pure films given in Fig.2 by saying that, contrary to what one might have expected, the greater expansion has been caused by the smaller probe group. We conclude that the behavior of such probe molecules is dictated not only by the size of attached group but by its nature. The pure film behavior clearly reflects the melting points of these compounds (stearic acid: 70° C, 12-(9-anthroyl)stearic acid: 79° C and 12-nitroxide stearic acid: 13° C). In addition, the equilibrium spreading pressure [11] of stearic acid is about 2 dynes/cm [12] while 12-(9-anthroyl) stearic acid only attains this value at about 30° C. In contrast, 12-nitroxide stearic acid, which melts at 13° C, must be spread as a liquid and thus the equilibrium spreading pressure is simply the film collapse pressure.

(b) Mixed Films

Interesting as the information concerning pure films of probe molecules may be, it does not allow us to clearly predict what the probe behavior will be like in a membrane-like environment. Clearly we must consider the behavior of these probe molecules in the presence of a host lipid, i.e., we must consider mixed monomolecular films containing small amounts of the probe. Our problems are not over, since in the film balance we measure an averaged-out behavior and the changes brought about by the introduction of probe molecules up to concentrations of 50:1, host:probe molecular ratio are very difficult to measure. Typical spin-labelling techniques call for actual concentrations of 100:1 or less, even although the probe may "see" a concentration of about 6:1. We have circumvented this difficulty by assuming that film balance concentrations of around 30 or 40:1 represent near ideal behavior and extrapolating this behavior to molar ratios of about 6:1 [2]. Following the example of Phillips and Chapman [13], we take a surface pressure of about 20 dynes/cm as producing a state of film molecule packing similar to that found in a liposome or membrane.

We will restrict ourselves here to reporting only our preliminary findings with mixed films. The detailed results will be presented later. Previously, we reported data on the mixed film system 12-nitroxide stearic acid/myristic acid, primarily at low probe concentrations [2]. Somewhat surprisingly, on completing our evaluation of this system by obtaining high probe concentration data, we found an interesting new aspect. Originally, only positive deviations from ideality had

been found at all surface pressures and low probe concentrations. Now, however, negative deviations were found at high probe concentrations and were shown to originate in the ability of the probe molecule to adopt a bent conformation. Between 80 and 90% myristic acid, however, appears sufficient to complete the erection of this molecule. In contrast, when dipalmitoyl lecithin was used as the host lipid, negative deviations extended to low probe concentrations at pressures as high as 16 dynes/cm. We are forced to conclude that, particularly at a lecithin interface, while the bulk of the 12-nitroxide stearic acid is in an erect conformation, a significant fraction may still be in a bent conformation. Since there is no reason to assume that it matters whether the host lipid has one or more hydrocarbons chains, we must conclude that the different behavior is a result of a specific interaction of the probe with the lecithin polar head group. Recently, evidence has been presented that this is, in fact, the case [14]. Our own results, both with pure [9] and mixed films, clearly indicate that this ability to adopt a bent conformation is very much dependent on the location of the oxazolidine ring on the carbon chan. Thus 5-nitroxide stearic acid has significantly stabilized conformation with both polar groups in the interface, an observation which may go a long way in explaining the bilayer water penetration profile of Griffith et al. [15].

Mixed film studies with 12-(9-anthroyl) stearic acid present a simpler but quite different picture. In mixed films with dipalmitoyl lecithin as the host lipid, as in pure films, 12-(9-anthroyl) stearic acid appears to adopt an erect conformation with the anthroyl group entirely in the hydrophobic region. Nevertheless, pronounced extensive negative deviations from ideal behavior were observed, and at no time were positive deviations found. The effects can only be interpreted in terms of a condensation of dipalmitoyl lecithin by 12-(9-anthroyl) stearic acid, since it is fully condensed at all pressures, and suggest that this particular probe will read too low a fluidity. While the picture is yet incomplete, moving the anthroyl group down the chain does not appear to change the behavior to any great extent, thus 2-(9-anthroyl)palmitic acid, in spite of the shorter alkane chain, still shows only negative deviations from ideality with dipalmitoyl lecithin films at 40° C.

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