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INTERACTIONS OF SPIN-LABELED LIPID MOLECULES WITH NATURAL LIPIDS IN MONOLAYERS AT THE AIR–WATER INTERFACE

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SUMMARY

The pressure–area curves of two widely-used spin-labeled lipids, 3-nitroxide cholestane and the acid form of 12-nitroxide stearate, were measured at the air-water interface. The 3-nitroxide cholestane molecule produced a curve similar to that of cholesterol or dihydrocholesterol (cholestanol), but the area per molecule was somewhat greater than those of the natural sterols. 12-Nitroxide stearate produced a very expanded pressure–area curve.

Mixed monolayers containing 3-nitroxide cholestane and either cholesterol or 1-palmitoyl-2-oleoyllecithin were measured to determine possible interactions, and these were compared with mixed monolayers containing cholesterol *plus* 1-palmitoyl-2-oleoyllecithin, or dihydrocholesterol *plus* this lecithin. The 3-nitroxide cholestane molecule interacts with cholesterol in such a manner as to expand the mixed films; however, this spin label exhibits little interaction with lecithin. In contrast, both cholesterol and dihydrocholesterol condensed lecithin films.

The application of this information to the interpretation of spin-label studies on lipid systems of membranes or membrane models is discussed.

INTRODUCTION

Much has been learned about the physical environment within phospholipid multilayers[1,2] or even intact membranes[3,4] by the use of spin-labeled lipid analogs. ESR spectra obtained from these spin-probe molecules yield valuable information about molecular motion and local environment which allows several inferences to be made about the molecular matrix. Some of these are solvent polarity, solvent viscosity, spatial orientation, effective radius of a spin-label matrix complex, Arrhenius activation energies, anisotropic motion, translational diffusion, and certain distance parameters. The accuracy and validity of the interpretation depend on experimental design, correct data analysis and proper choice of spin label. It should be recognized that the probe group is rather bulky and may itself influence its environment, and it may not arrange itself within the matrix structure exactly as the unlabeled analog does.

Therefore, it seemed worthwhile to examine the behavior of spin-labeled lipid molecules in monomolecular layers on water, in order to see how a spin-labeled mole-

cule would orient at a polar–nonpolar interface. It would also be valuable to measure interactions between spin-labeled lipid molecules and some of the natural components of membranes or membrane model systems. Cadenhead and colleagues[12,13] have studied the surface properties of 3-nitroxide cholestane and its interactions with myristic acid, and have compared the behavior of this spin-labeled lipid with that of cholesterol. Since unesterified myristic acid is not normally a major component of membranes, we decided to examine the interactions of 3-nitroxide cholestane with a synthetic lecithin and with cholesterol because these lipids are common components of natural membranes. Any interactions that we might find would therefore be directly relevant to the data obtained by ESR studies employing this probe molecule in membranes or multilayer lipid systems containing cholesterol and lecithins. Also, we planned to study the behavior of spin-labeled stearic acid, because this probe is commonly used in ESR studies of membranes. Accordingly, spin-labeled cholestanol and stearic acid were studied at the air–water interface. Their behavior was also compared with that of the unlabeled analogs, cholesterol, cholestanol (dihydrocholesterol), stearic acid, and oleic acid. Spin-labeled cholestanol was also studied in mixtures with cholesterol or 1-palmitoyl-2-oleoyl lecithin (the major component in egg lecithins) since these latter two lipids are components of most natural membranes, and they or similar lipids have been used in many spin-label studies.

MATERIALS AND METHODS

Spin-labeled cholestanol (3-nitroxide cholestane or 3-spiro [2′-(*N*-oxyl-4′, 4′-dimethyloxazolidine)]cholestane) was synthesized by the method of Keana *et al.*[5]. The product was purified by thin-layer chromatography on Silica Gel G (Merck) using chloroform as the developing solvent. No attempt to separate epimers was made. Its melting point was 174 °C (lit. 175–176 °C)[5]. Calculated for $C_{31}H_{54}O_2N$: C, 78.76 %; H, 11.51 %; N, 2.96 %. Found: C, 78.79 %; H, 10.69 %; N, 2.91 %.

Spin-labeled stearic acid (12-nitroxide stearic acid) was prepared as described by Waggoner *et al.*[6]. It was purified by thin-layer chromatography on Silica Gel H (Merck) with chloroform–methanol water (195:75:12, v/v/v) as developing solvent. Its melting point, which is near room temperature, was not measured. Calculated for $C_{22}H_{42}O_4N$: C, 68.69 %; H, 11.01 %; N, 3.64 %;Found: C, 68.31 %; H, 10.62 %; N, 3.65 %.

Lecithin (1-palmitoyl-2-oleoyllecithin) was synthesized and purified by a procedure described previously[7]. The mole ratio of oleic acid to palmitic acid was 1.05:1.00, as determined by gas chromatography[7].

Stearic and oleic acids were obtained from Hormel Institute (Austin, Minn.) and were stated to be at least 99 % pure. The methyl esters of these were analyzed by gas chromatography and no contaminants comprising greater than a total of 1 % were found.

Cholesterol (Nutritional Biochemicals Corp., standard for clinical work, Cleveland, Ohio) was examined for purity by thin-layer chromatography[8,9]; only one component was detected. Dihydrocholesterol (cholestanol) (Calbiochem, San Diego, Calif.) was recrystallized from absolute ethanol and tested by thin-layer chromatography, as above; no other components were detected. The melting point of cholesterol

was 148 °C (lit. 148.5 and 149 °C[10,11]) and the melting point of dihydrocholesterol was 141 °C (lit. 142 °C[11]).

The sterols or spin-labeled lipids which were to be spread at the air–water interface were weighed after the compounds had been dried 12 h or more over P_2O_5 under vacuum at room temperature. Mixtures of redistilled reagent-grade benzene and chloroform were used to dissolve the lipids. The concentrations of the fatty acid solutions and the lecithin solutions were determined by gas chomatography, after the preparation of methyl esters[7].

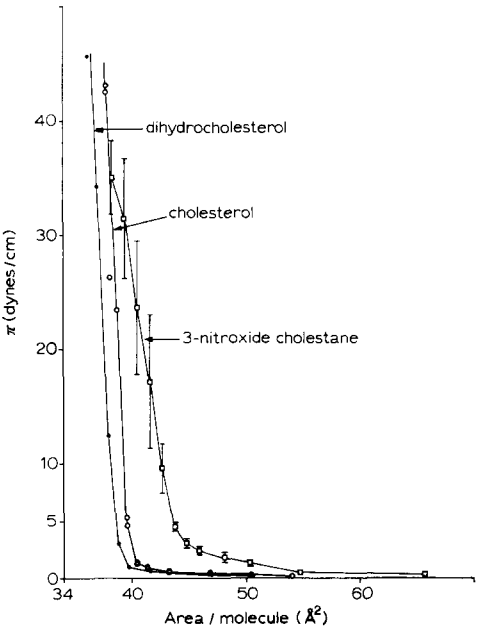
Solutions of pure lipids and mixtures of lipids were applied with a Hamilton syringe to the water in a surface balance (Cenco Hydrophil Balance, Central Scientific Co., Chicago,Ill) at 22 ± 1 °C, as described before[7]. The films were compressed by moving the barrier by hand; each reading took about 10–15 s, and an entire curve would require 10–15 min for measurement. Replicate measurements made on the same day agreed within ± 1 Å^2, and a complete set of mixtures was always measured on the same day. Duplicate runs on different days agreed within ± 3 Å^2 or less (<5%). Each curve was measured twice or more.

The films were spread on distilled water, at a pH of about 5.2, except for one set of measurements carried out on 3-nitroxide cholestane which were done on 0.01 M HCl, 2 M NaCl (not shown). These latter determinations were done both in an effort to reproduce the pressure–area curve reported by Cadenhead and Katti[12] and to determine whether the spin-labeled molecule would undergo spin loss at the low pH since the nitroxide group is known to be sensitive to acid. The curve was very similar to that found with distilled water as subphase. The 3-nitroxide cholestane spreading solution, as well as that of 12-nitroxide stearate was qualitatively analyzed for the presence of spin before spreading (X-band electron paramagnetic resonance spectrometer, Model JES-ME-1X, Japan Electron Optics). Part of the 3-nitroxide cholestane film spread on the acidic subphase was recovered, and was found to retain its spin signal. These experiments indicated that the spin label had survived exposure to acid. The discrepancy between our data and the pressure–area curve of 3-nitroxide cholestanol first reported by Cadenhead and Katti[12] is due to an error (impure sample) in their first measurement. This is fully explained in a second report[13] and their corrected curve[13] is very similar to the one shown here for 3-nitroxide cholestane.

RESULTS

Pressure–area curves of cholesterol, dihydrocholesterol (cholestanol) and 3-nitroxide cholestane are shown in Fig. 1. The curve exhibited by 3-nitroxide cholestane resemblestho se of the natural sterols, but the areas per molecule are somewhat larger. This compound formed an unstable monolayer which could barely withstand compression much above 5 dynes/cm. As a consequence of this instability, the data from four films were combined and the curve shows the standard errors of these measurements. The other films were stable.

Cholesterol is a common component of natural membranes or membrane model systems. If 3-nitroxide cholestanol is a perfect analog for cholesterol, these molecules should form an ideal mixture. It is therefore important to determine whether cholesterol interacts with 3-nitroxide cholestane or whether an ideal mixture is formed. When monolayers containing both 3-nitroxide cholestane and cholesterol were mea-



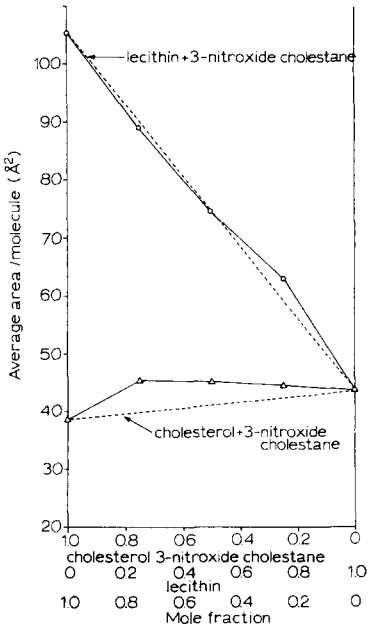


Fig. 1. Pressure-area curves of cholesterol, dihydrocholesterol (cholestanol) and 3-nitroxide cholestane at the air–water interface. Standard errors in measurements of 3-nitroxide cholestane are indicated by vertical bars.

Fig. 2. Interactions of 3-nitroxide cholestane with cholesterol (lower curve) and with 1-palmitoyl-2-oleoyllecithin (upper curve) in monolayers at the air–water interface.

sured, an expansion of the film was observed (Fig. 2) which indicates that there is an interaction between the unlike molecules. Similar expansions were observed at higher pressures, but are not shown because the data for pure 3-nitroxide cholestane were not precise above 5 dynes/cm.

Lecithins are important components of most membranes and membrane model systems; furthermore, most natural lecithins are known to interact with cholesterol. It was therefore valuable to find whether 3-nitroxide cholestane would exhibit this type of interaction with a natural lecithin of known structure, and to compare the behavior of 3-nitroxide cholestane with that of natural sterols. Mixtures of 3-nitroxide cholestane with 1-palmitoyl-2-oleoyllecithin demonstrate that there is little interaction between 3-nitroxide cholestane and this lecithin in a monolayer (Fig. 2). Cholesterol condenses strongly with this lecithin (Fig. 3) as it does with many other lecithins of natural structure[7,14]. Dihydrocholesterol also condenses but to a lesser extent (Fig. 3). These data illustrate that the physical behavior at an interface of 3-nitroxide cholestane is distinctly different from that of cholesterol or dihydrocholesterol.

Another widely-used spin label, 12-nitroxide stearate, was measured for comparison with its natural analog, stearic acid, and with oleic acid as an example of a fatty acid containing an irregularity (double bond) which leads to formation of expanded monolayer films (Fig. 4) Stearic acid formed a compact film which could be compressed to a surface pressure of more than 40 dynes/cm. Oleic acid exhibited a

much more expanded film, whose curve leveled off at about 32 dynes/cm. It could be compressed for several more Å²/molecule without exerting increased pressure on the mica float. In contrast, 12-nitroxide stearate produced an expanded film which occupied more than 130 Å² at low pressures (< 2 dynes/cm). As the pressure was increased, the films gradually declined in area to about 20 Å², at which point the film pressure did not increase despite additional compression. The maximum pressure attainable was about 28 dynes/cm. These observations indicate that 12-nitroxide

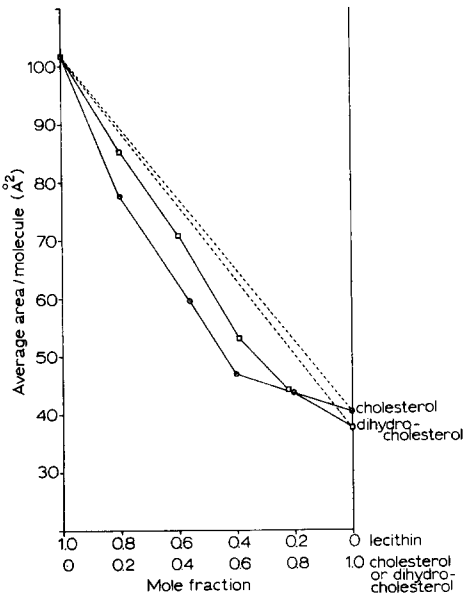


Fig. 3. Interactions of 1-palmitoyl-2-oleoyllecithin with cholesterol and with dihydrocholesterol at the air–water interface.

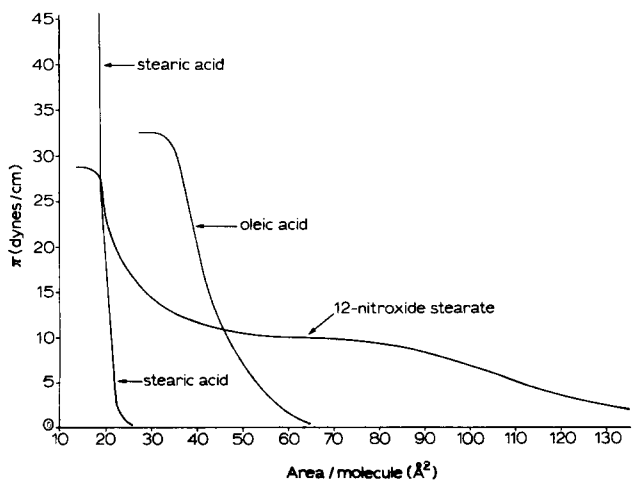


Fig. 4. Pressure–area curves of oleic acid, stearic acid and 12-nitroxide stearic acid at the air–water interface.

stearate does not resemble either stearic acid or oleic acid in their behavior at a polar–nonpolar interface.

DISCUSSION

Hydrocarbon molecules containing a nitroxide orient at a polar–nonpolar interface when the nitroxide group acts as the polar moiety. This observation is illustrated by the formation of an unstable monolayer of 3-nitroxide cholestane where the nitroxide is the only polar group. The instability of this film suggests that the polarity of the nitroxide group is considerably weaker than that of a hydroxyl group, and may be comparable to that of a ketone, since 5-cholestene-3-one produces a pressure–area curve similar to that of 3-nitroxide cholestane[15].

We attribute the large areas formed by 12-nitroxide stearate to the localization of both polar groups at the air–water interface. The large area occupied by each 12-nitroxide stearate molecule suggests that the stearic acid backbone is oriented horizontally for a considerable distance. Approximations made from Pauling–Corey space-filling models show that the 12-nitroxide stearate molecule would be expected to occupy about 110 Å^2 molecule if the chains are tightly packed in a horizontal orientation. If the carboxyl group of 12-nitroxide stearate is at the interface and the rest of the chain is rigidly oriented perpendicular to the plane of the interface, the minimum cross-sectional area should be about 36 Å^2. However, the film can be compressed even below this area, to an average area of about 20 Å^2/molecule which indicates that some of the molecules are no longer in a monomolecular layer.

It is interesting that the nitroxide group itself can orient a molecule at a polar–nonpolar interface, since ESR measurements of phospholipid multilayers indicate that spin-labeled fatty acids orient with their carboxyl groups localized at the polar boundaries[1]. The 3-nitroxide cholestane molecule also orients in multilayers, with the nitroxide group at the interface and the long axis of the molecule perpendicular to the plane of the multilayer[1]. In this case the orientation could have been caused by a geometric fit between the rigid steroid nucleus and the other lipids, by the polarity of the nitroxide group, or both. The present measurements therefore provide additional information about the forces that may be responsible for the orientation of these molecules; namely the polarity of the nitroxide group. It is possible that 12-nitroxide stearate, which appears to have both polar groups near the polar phase in a monolayer, may also behave similarly in multilayer systems under certain conditions.

Cadenhead and colleagues[12,13] have raised the important question, are spin-labeled lipids adequate analogs for the natural molecules? The answer does not seem to be a simple yes or no. Our data show, for example, that 3-nitroxide cholestane is not as firmly attached as cholesterol or dihydrocholesterol to the polar boundary (unstable films) and that it is probably not oriented at the same angle as the natural sterols are, since the molecules occupy more area per molecule than the natural sterols do. Furthermore, an expansion of the film occurs when cholesterol and 3-nitroxide cholestanol are spread in mixed films. This phenomenon indicates that unlike molecules have greater affinity for each other than like molecules do; because, if the cholesterol molecules associated with each other, excluding the 3-nitroxide cholestane, the film would consist of regions of pure cholesterol and other regions of

pure 3-nitroxide cholestane. Such a film would occupy an area equal to the sum of the areas of the individual components, which is not the case in mixed films of cholesterol and 3-nitroxide cholestane. Finally, 3-nitroxide cholestane does not behave as cholesterol or dihydrocholesterol do with a lecithin. These phenomena show that 3-nitroxide cholestane clearly is not a perfect analog of either cholesterol or dihydrocholesterol. However, it is a fairly good analog—the nitroxide group is polar enough to orient the sterol structure in an almost normal position at an interface. The fact that spin-labeled lipids may specifically interact with a particular membrane component is especially important because this information should allow one to interpret the ESR spectra in greater detail. The knowledge that 3-nitroxide cholestane seems to have a special affinity for cholesterol rather than the lecithin (at least in monolayers) may allow the ESR data to be interpreted more precisely when 3-nitroxide cholestane is used in multilayer systems containing cholesterol and lecithin. In the future, it might be feasible to select a spin-labeled lipid that has been found to interact with a specific membrane molecule in order to study this particular molecule in the presence of many others.

These observations suggest that the value of ESR data could be increased by examination of the properties of ESR probes in monolayers, especially in mixtures with known membrane components. The behavior at interfaces of the probes themselves is valuable information, because it should lead to better knowledge of the location of the probe in analogous multilayer systems; and information concerning the presence and strength of interactions between the probes and normal membrane components should greatly aid in the interpretation of ESR spectra obtained from the more complex multilayer systems.

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