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A MICROVISCOSITY BARRIER IN THE LIPID BILAYER DUE TO THE PRESENCE OF PHOSPHOLIPIDS CONTAINING UNSATURATED ACYL CHAINS

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SUMMARY

A series of fluorescent probes which locate a graded series of depths from the surface to the centre of the lipid bilayer have been used to measure the fluidity gradient in liposomes and natural membranes. In dioleoyl phosphatidylcholine liposomes and in cells which have a high content of unsaturated phospholipids, a region of high microviscosity is detected near the cis double bond/s. The significance of this phenomenon is discussed in terms of the penetration and lateral movement of membrane protein.

INTRODUCTION

Biomembranes need to be in a fluid state in order to maintain complete biological function. Recently, Seelig and Seelig (1) have recognized that the term "fluidity" as applied to the lipid bilayer, encompasses two concepts which under certain circumstances may be quite independent. Firstly, there is the concept of packing which describes the degree to which phospholipid acyl chains may approach each other to within van der Waal's distances. Thus the occurrence of a single cis double bond in an acyl chain increases the area per molecule projected at the bilayer surface and decreases order to the extent that the phase transition temperature is greatly reduced. Secondly, fluidity refers to the rates of anisotropic motion (segmental rotation and lateral diffusion) of phospholipid molecules. At any one temperature both the rate and amplitude

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of the segmental motion of acyl carbon atoms increases as the terminal methyl group is approached such that there is a gradient a fluidity from the surface to the centre of the bilayer.

In this report we describe the use of fluorescence polarization to determine the fluidity at different depths in the bilayer using a set of fluorescent fatty acid probes. The results indicate that the microviscosity in the region of the double bond is significantly higher than in regions nearer the surface or deeper in the bilayer. Evidence is presented that such a situation may also occur in natural membranes.

MATERIALS AND METHODS

n-(-Anthroyloxy) fatty acids were prepared from anthracene-9-carboxylic acid and the appropriate hydroxy fatty acid by anhydride synthesis (2,3) and purified by preparative thin-layer chromatography. Methyl-9-anthroate was prepared by the method of Parish and Stock (4). 1,2-Distearoyl phosphatidylcholine and 1,2-dioleoyl phosphatidylcholine were purchased from Serdary Laboratories. 1,2-Dimyristoyl phosphatidylcholine was synthesized in this laboratory (5). Liposomes were prepared according to usual procedures and were sonicated for 3 min under N_2. Fluorescent lifetimes (τ) of probes located in the bilayers were measured using the technique of single photon counting (337 nm excitation, >420 nm emission) with vertically polarized exciting light and the emission polarizer set at 54.7° to the vertical plane to eliminate the effects of polarized emission. Values of the limiting polarization (p_o) were measured in glycerol solutions (6) and were the same for all probes to within experimental error ($p_o = 0.29 + 0.01$). Polarization measurements were made with a Perkin-Elmer MPF-3 spectrofluorometer equipped with polaroid filters. Some measurements were also made with an instrument constructed in this laboratory to the design of Bashford et al. (2). Results were analysed according to the Perrin equation (7) which relates measured polarization to the rotational relaxation time (ρ) of the fluorophore. Although this equation applies only to isotropic rotation of a fluorophore and is not strictly applicable to the anisotropic rotation of the probes in lipid bilayers it does offer some correction for the variation of lifetime. In fact this variation is small and gradients in polarization were therefore similar to gradients in rotational relaxation time.

The position of each probe in dimyristoyl phosphatidylcholine bilayers was verified by measuring the accessibility of the fluorophores to Cu(II), a paramagnetic quenching agent. These results will be presented elsewhere. The order of accessibility was 2-AP > 6-AS > 9-AS > 12-AS > M-9-A. On the

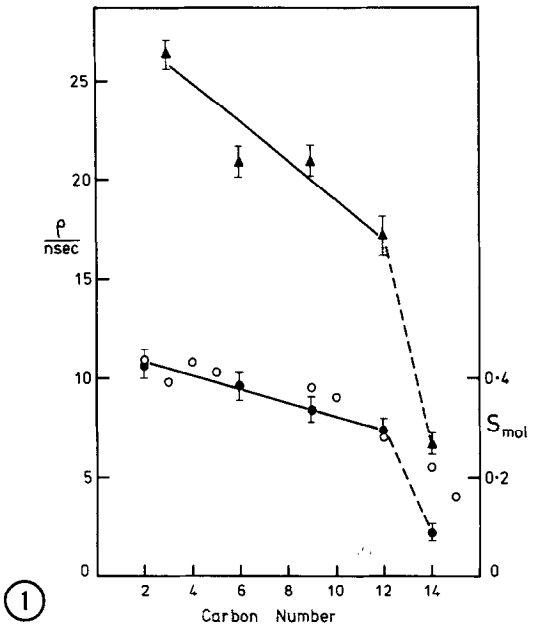
Abbreviations: 2-AP, 2-(9-anthroyloxy) palmitic acid; n-AS (n = 6, 9, 12), n-(9-anthroyloxy) stearic acid; M-9-A, methyl-9-anthroate.

basis of these results we have placed M-9-A close to C-14 in the dimyristoyl phosphatidylcholine system.

Cultured mosquito cells (Aedes albopictus) were the gift of Dr. L. Dalgarno. Mouse thymocytes were prepared by the method of Boyum (8).

RESULTS AND DISCUSSION

The rotational relaxation times of the probes in dimyristoyl phosphatidylcholine liposomes above and below the phase transition temperature are shown in Fig. 1. As the fluorophore is moved further along the acyl chain there is a decrease in ρ reflecting an increase in bilayer fluidity resulting from an increase in the probability of trans-gauche isomerizations along the acyl chains of the phospholipids (9). A notable feature is the very sharp decrease in ρ between C-12 and C-14. The rotational characteristics of M-9-A may not be directly comparable with the fatty acid probes since it is not attached to an acyl chain. Nevertheless, it is noteworthy that other physical methods record a very steep increase in chain mobility as the terminal methyl group is approached. For example, Seelig and Seelig (9) have determined an order parameter from the quadrupole splitting of deuterium labelled dipalmitoyl phosphatidylcholine and their results for a temperature slightly above the phase transition temperature are included in Fig. 2 for comparison. Studies of ^{13}C spin-lattice relaxation times have yielded similar results (10). These methods measure the angular fluctuations of the chain segments and are essentially techniques which do not perturb the bilayer structure. It might be expected that the bulky anthracene fluorophore would distort the bilayer structure. However, the good agreement with the deuterium magnetic resonance results would suggest that this effect is minimal possibly due to the favourable molar ratio of probe to phospholipid (1:400) employed in these experiments. Certainly,



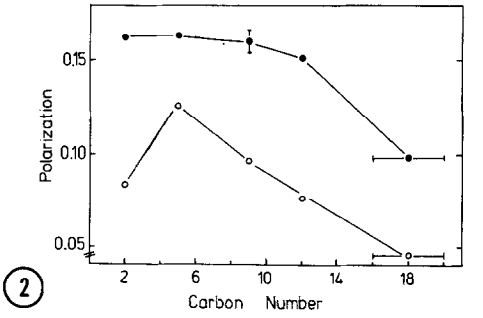


Fig. 1. The dependence of the rotational relaxation time (ρ) on the position at which the anthroyloxy group is attached to the acyl chain of the fatty acid probe for dimyristoyl phosphatidylcholine liposomes at temperatures above (○, 27°) and below (△, 21°) the phase transition temperature (24°). The concentration of phospholipid and probe was 0.52 mM and 1.3 μM, respectively. Separate binding experiments indicated that at this phospholipid:probe ratio all probe molecules were bound to the bilayers. The variation of τ with temperature was determined from the relation $I_x/\tau_x = I_y/\tau_y$ where I is the fluorescence intensity and the subscripts refer to two different temperatures. Vertical error bars indicate error due to the determination of polarization and lifetime.

Fig. 2. The dependence of fluorescence polarization on the position of probes in distearoyl phosphatidylcholine (●) and dioleoyl phosphatidylcholine liposomes (▲). The lipid concentration was 0.5 mM. The vertical error bars are as described for Fig. 1. The horizontal error bar for M-9-A indicates the uncertaintly in the position of this probe at the centre of the bilayer.

the fluorescent fatty acids report the correct phase transition temperature for dimyristoyl phosphatidylcholine.

The influence of a cis double bond on bilayer structure has been reported recently by Seelig and Seelig (1). These authors reported the influence of an oleoyl chain at position 2

of the glycerol backbone on a selectively deuterated palmitoyl chain at position 1 in terms of the deuterium order parameter (S_{mol}). The order parameter increased from C-2 to C-6 of the palmitoyl chain and then showed a steady and relatively steep decline. The position of the maximum at C-6 of the palmitoyl chain when the double bond on the oleoyl chain was located between carbons 9 and 10 was explained in terms of the bend which occurs in the oleoyl chain close to the glycerol backbone which brings segments of that chain closer to the bilayer surface. The results were interpreted as indicating the local stiffening effect created by the presence of a cis double bond in the adjacent acyl chain.

We now report a similar effect using the fluorescence depolarization technique. Fig. 2 compares the polarization profiles for distearoyl and dioleoyl phosphatidylcholine. The profile for the unsaturated lecithin is characterized by a maximum near C-6. The position of this maximum is unusual since the arguments outlined earlier with respect to the deuterium NMR experiment (1) cannot be applied in the case of the fluorescent fatty acid probes. For dioleoyl phosphatidylcholine some averaging of the stiffening effect of the double bond might be expected due to the unequal configuration of carbon chains near positions 1 and 2 of the glycerol backbone but this is insufficient to account for the results. The data suggest that in the case of the fluorescent probe technique, the effect of the cis double bond is manifest above the actual position in the acyl chain.

The polarization technique cannot distinguish between a decrease in microviscosity caused by increased motion within lipid molecules, or a decrease in microviscosity due to

increased molecular separation. In this latter connection, we can expect that an increase in the separation between head groups in the unsaturated phospholipid bilayer (11) provides a less restricted environment for the rotation of the fluorescent probe. It is clear however that the statement that "unsaturated phospholipids increase the fluidity of the bilayer" is an incomplete description of their effect since that effect varies with bilayer depth.

We have observed similar polarization profiles for some natural membranes. Fig. 3 shows the profiles for mouse thymocytes and cultured mosquito cells. Although this characteristic in natural membranes may be affected or modified by membrane protein we make the following comments. The mosquito cells have a low cholesterol content (<3% total lipid) and an unusually high content of monoenoic fatty acids (16:1 and 18:1) in their glycero-phospholipids (12). To our knowledge the fatty acid compositions of the thymocyte phospholipids has not been determined. However by analogy with lymphocytes from the pig mesenteric lymph node (13) we might expect relatively large amounts of 18:1 fatty acid. It would therefore appear that significant amounts of unsaturated phospholipid in the plasma membrane may lead to a maximum in the polarization profile. The similarity of this profile with that obtained for liposomes of dioleoyl phosphatidylcholine strongly suggests that unsaturation of the phospholipid acyl chains is the cause of the phenomenon. We have not observed a maximum in the profile for erythrocyte ghost membranes. However, this need not be related to its high cholesterol phospholipid ratio. Increasing the proportion of cholesterol in dioleoyl phosphatidylcholine liposomes did not change the shape of the polarization profile

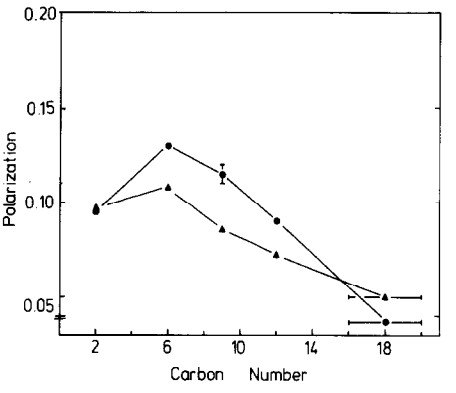


Fig. 3. The dependence of fluorescence polarization on probe position. ●, Mouse thymocytes suspended in Tris-HCl buffer (0.05 M Tris, 0.1 M KCl, pH 7.5), 10^6 cells/ml. ▲, Cultured mosquito cells in 0.13 M NaCl, 0.005 M KCl, 0.004 M $NaHCO_3$, 0.1% glucose, pH 7.4, 10^5 cells/ml. On completion of the experiment all thymocytes were trypan blue positive. Error bars are as described in Fig. 2.

depicted in Fig. 2 but simply shifted it to higher values. It seems more likely that the appearance of a maximum in the polarization profiles of natural membranes is indicative of laterally segregated areas rich in unsaturated phospholipid into which the probe preferentially localizes. It is known for example (2), that in laterally segregated mixtures of dioleoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine the C-12 probe preferentially localizes in the more fluid phase (i.e. dioleoyl phosphatidylcholine).

Finally, we note that the existence of a microviscosity barrier in each leaflet of the bilayer in the vicinity of the double bonds may determine the degree of penetration and the rate of lateral diffusion of membrane proteins. For example, if extrinsic membrane protein is unable to penetrate into a a more viscous environment near double bonds it may have greater lateral mobility than intrinsic protein which must penetrate

this barrier. To this effect must be added the modifying influence of cholesterol which recent experiments suggest can also control the penetration of extrinsic protein (14). It thus appears that the hydrocarbon region of lipid bilayers may well play a significant role in determining protein-lipid interactions other than just providing the hydrophobic matrix in which membrane proteins can function.

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