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ALIPHATIC CHAIN TRANSITIONS OF PHOSPHOLIPID VESICLES AND PHOSPHOLIPID DISPERSIONS DETERMINED BY POLARIZATION OF FLUORESCENCE

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SUMMARY

The polarization of fluorescence of dansyl phosphatidylethanolamine and 9-methylanthracene shows that these compounds are reliable indicators of the order–disorder transitions of the phospholipid aliphatic chains in bilayer systems. The transition is better defined in phospholipid dispersions than in vesicles. It is concluded that the two models are not identical as far as the structure near the melting temperature is concerned. Experiments in turbid solutions were performed with horizontal slits either in the incident or emitted beams, which eliminate the effect of light scattering. This improvement in experimental technique may facilitate the fluorescence polarization study of membrane suspensions.

INTRODUCTION

The use of well-defined model systems and the application of techniques, which provide insight into the conformational state of membrane components, may facilitate understanding at the molecular level of the diversity of functions of membranes. Lipid dispersions have been extensively studied (see for example refs 1–3). The closed vesicles which result from prolongated sonication of these dispersions have an aqueous compartment and a bilayer membrane and furthermore can be carefully calibrated[4]. As a technique to look into the dynamic structure of the system, fluorescence polarization appears promising because the polarization properties of chromophores provide information concerning the fluidity of their immediate environment, but this technique is more informative when chromophores are specifically located inside the bilayer. One of the most widely used chromophores is 1-anilino-8-naphthalenesulfonate (ANS). Its precise location in the systems is not easily assessed. It is possible to avoid this uncertainty by using a label covalently bound to one of the membrane components. Dansyl phosphatidylethanolamine consists of the dansyl chromophore attached to the phosphatidylethanolamine moiety and it probes the glycerol region of the bilayer[5]. In order to test its fluorescence properties certain conditions must be fulfilled.

(a) Polarization measurements requiring clear suspension, solutions of vesicles are convenient because they do not show any turbidity when the phospholipid

Abbreviation: ANS, 1-anilino-8-naphthalenesulfonate.

concentration is around 1 mg/ml. But, as we will see below, it is possible in some cases to study turbid solutions.

(b) Depolarization of fluorescence from small labeled vesicles (diameter 250–300 Å) results from two superimposed contributions: the intrinsic rotational motion of the chromophore within the bilayer and the rotational diffusion of the whole vesicle. We have shown[6] that this latter contribution is negligible, at least when the lifetime of the chromophore excited state is about 15 ns. This is important, since only the former effect is directly correlated to a change in the fluidity of the chromophore environment. We report here the study of the aliphatic chain transitions of phospholipid vesicles and phospholipid aqueous dispersions labeled with fluorescent probes; namely, ANS, 9-methylanthracene and dansyl phosphatidylethanolamine.

MATERIALS AND METHODS

Materials

All reagents were A.R. grade and were used without further purification. The water was distilled and deionized. Aluminium oxide was obtained from Rhône Poulenc, Sepharose 4B from Fine Chemical Pharmacia silicic acid from Mallinckrodt, dipalmitoylphosphatidylethanolamine from Fluka, dipalmitoylphosphatidylcholine from N.B.C., dimyristoylphosphatidylcholine from Koch–Light Laboratory and dansyl chloride from Baker.

Egg phosphatidylcholine

Crude phosphatidylcholine was extracted from hen's egg yolk according to the method of Singleton *et al.*[7], purified in an aluminium oxide column (elution with chloroform–methanol, 1 : 1, v/v) and checked for purity by thin-layer chromatography. The purified phospholipid was dissolved in benzene, lyophilized and stored under vacuum at −20 °C.

Synthesis of the dansyl phosphatidylethanolamine

The process used was essentially that of Shechter *et al.*[8] with some minor modifications. L-Dipalmitoylethanolamine, dansyl chloride and dry triethylamine were dissolved in benzene and shaken for 6 h at 45 °C. The reaction was then almost complete as seen by thin-layer chromatography. The mixture was carefully washed with dilute solutions of bicarbonate and HCl, then the solution was dried and evaporated. The pale yellow compound obtained was dissolved in diethyl ether and precipitated with acetone between −15 and −20 °C in a cold room. The product still contained a fluorescent lyso derivate as indicated by chromatographic analysis. Pure dansyl phosphatidylethanolamine was then obtained by preparative thin-layer chromatography on silicic acid (elution solution, chloroform–methanol–water 65:25:4, v/v). The compound was dissolved in benzene, lyophilized and stored in sealed ampullae.

Preparation of labeled samples

Different methods of labeling were used.

(a) ANS was simply added to the solution.

(b) Dansyl phosphatidylethanolamine and phospholipids (molar ratio 1/100)

were mixed with chloroform which was then removed under reduced pressure. This process was repeated several times before the addition of aqueous solution. These solutions were studied (phospholipid dispersions) or used to prepare the vesicles. To this end, the swelling solutions were ultrasonically irradiated to clearness under N_2, with an Annemasse F 50 sonifier. The sonicate was passed through a Sepharose 4B column and the elution diagrams automatically recorded at 300 nm. The elution profiles were similar to those described elsewhere, with the peak of large particles and that of vesicles of diameter 250–300 Å (ref. 4). The concentrations of solutions determined by phosphorus titration after $HClO_4$ hydrolysis were always about 1 mg of phospholipid per ml.

(c) We were unable to label the vesicles with 9-methylanthracene by the previous method because separation of the probe and vesicles occurs in the column. We have also followed procedures derived from that of Shinitzky *et al.*[9] for labeling micelles with 2-methylanthracene. In the first method, the labeling was carried out by mixing an acetone solution of the probe with the vesicles solution under vigorous stirring. The final acetone concentration was very low (about 1%) and it can be further assumed that the acetone does not perturb the bilayer. In the second method, the acetone solution was poured on sintered glass which was dried and then washed several times with water till the fluorescence was barely detectable in water. Then the vesicle solution was passed through sintered glass. To assure a homogeneous distribution of the probe, the solutions were equilibrated 24 h before use.

Fluorescence measurements

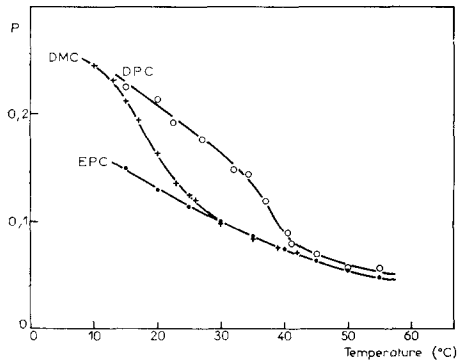
The degree of polarization $P=(I_{/\!/}-I_{\perp})/(I_{/\!/}+I_{\perp})$ was determined with an apparatus built in the laboratory. The excitation wavelength was 366 nm and the fluorescence observed above 450 nm. The exciting light was polarized vertically. In fluorescence–polarization measurements, the main source of error is due to scattering of the polarized exciting light, which results in an increase in the measured degree of polarization; this effect was eliminated with an interference filter. Another source of error with turbid solutions is due to scattering of fluorescent light, which reduces the degree of polarization. As the solutions containing the vesicles are in all cases very clear, this latter effect is very small, being less than 1%. To study the phospholipid dispersions, we used the system of horizontal slits of Teale[10].

RESULTS

Vesicles[11]

In Fig. 1, the degree of polarization (P) of the dansyl phosphatidylethanolamine located in egg phosphatidylcholine, dimyristoylphosphatidylcholine, or dipalmitoylphosphatidylcholine vesicles, is plotted as a function of temperature. P is significantly greater for dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine vesicles at low temperature, but it decreases quickly near 20 °C for the former and 40 °C for the latter systems. As Ladbrooke and Chapman[3] have demonstrated, the aqueous dispersions of egg phosphatidylcholine do not show any transition in the 10–55 °C temperature range (the transition is about −10 °C), but dipalmitoyl- and dimyristoylphosphatidylcholine exhibit a large endothermic order–disorder transition of their aliphatic chains at 41 °C and 23 °C, respectively. Thus it may be

inferred that the dansyl phosphatidylethanolamine probe detects the transitions. According to Waggoner and Stryer[5], the dansyl chromophore probes the glycerol region of the bilayer and our findings suggest that the aliphatic chain transitions are observed because the spacing between phospholipid molecules increases slightly



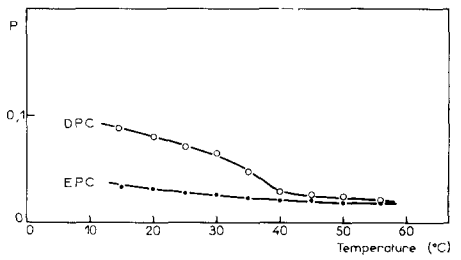


Fig. 1. Effect of temperature on the degree of polarization (*P*) of dansyl phosphatidylethanolamine inserted in vesicles of: ●—●, egg phosphatidylcholine (EPC); +—+, dimyristoylphosphatidylcholine (DMC) and ○—○, dipalmitoylphosphatidylcholine (DPC).

Fig. 2. Effect of temperature on the degree of polarization (*P*) of 9-methylanthracene inserted in vesicles of: ●—●, egg phosphatidylcholine (EPC) and ○—○, dipalmitoylphosphatidylcholine (DPC).

when melting occurs, allowing a greater mobility of the chromophore. Moreover, it is interesting to note that the values of *P* above the transition temperature are practically identical for the three systems. This result means that the fluidity of the probe microenvironment is the same and does not depend on the chain lengths. With 9-methylanthracene, the curves (Fig. 2) are similar to those obtained with dansyl phosphatidylethanolamine. 9-Methylanthracene certainly probes the hydrocarbon matrix and, as might be expected, detects the transition. Here again the curves are identical above the transition temperature. In contrast with the previous probes the degree of polarization of ANS (Fig. 3) decreases progressively when the temperature increases from 10–55 °C. *P* always remains higher for dipalmitoylphosphatidylcholine than for egg phosphatidylcholine. Neither of the curves shows any discontinuity; therefore, within the accuracy of our measurements, we are unable to detect phase transitions of phospholipids. This result is consistent with the location of ANS in the polar head region of the bilayer which has been recently determined by X-ray diffraction[12] and NMR spectroscopy[13].

Phospholipid dispersions

The phospholipid dispersions are very turbid and become milky at high concentration, indicating an important light-scattering effect. Scattering of the emitted light leads to an apparent decrease in the degree of polarization. Teale[10] has shown theoretically and experimentally that the depolarization of the emitted radiation is strongly dependent on the geometry of the fluorescent sample and can be reduced using horizontal 1-mm wide slits. In order to test whether the slits have this expected effect on our systems, we started with an optically clear solution of egg phosphatidyl-

choline vesicles labeled with dansyl phosphatidylethanolamine and increased progressively the turbidity of the sample by addition of a slightly sonicated egg phosphatidylcholine aqueous dispersion. The degree of polarization was then plotted as a function of the solution absorbance at 366 nm. It should be noted that the dansyl

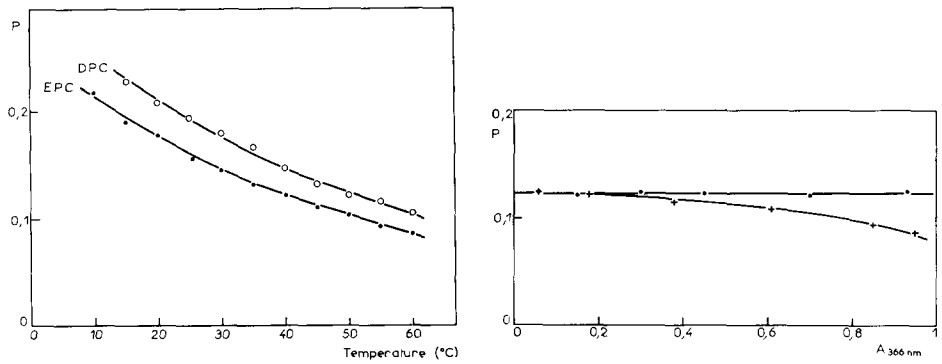


Fig. 3. Effect of temperature on the degree of polarization (P) of 1-anilino-8-naphthalene sulfonate inserted in vesicles of: ●—●, egg phosphatidylcholine (EPC) and ○—○, dipalmitoylphosphatidylcholine (DPC).

Fig. 4. Effect of light scattering on the degree of polarization (P) of dansyl phosphatidylethanolamine inserted in egg phosphatidylcholine (EPC) vesicles *versus* the turbidity measured at 366 nm, temperature 20 °C. The turbidity is gradually changed by addition of a slightly sonicated aqueous EPC dispersion; (a) ●—● with slits 1-mm wide; (b) +—+ without slits.

group absorbs at 366 nm but its concentration is very small and therefore the absorbance is only a function of sample turbidity. From Fig. 4, it can be seen that the decrease of P is about 30% when the absorbance increases from 0–0.9. The results are markedly different and the degree of polarization remains constant when horizontal slits are placed on the sample on the incident and emitted beams. All experiments on phospholipid dispersions were performed with this modification. If one compares the results reported in Fig. 5 with those obtained with vesicles, one notices that the curves are similar below and above the transition temperature. Thus it may be concluded that the use of slits enables the determination of the absolute degree of polarization of phospholipid dispersions.

Relationship between P and the mobility of the probe

P is a function of the lifetime τ of the excited molecule and of the microviscosity of the chromophore environment $P=f(\eta/\tau)$. Weber[14] has recently derived a theory for the relationship between fluorescence depolarization and Brownian rotations of non-spherical particles. For the more common case of rotating plates (aromatic molecules) in which the oscillators of absorption and emission are coplanar, and if only small rotations can take place between excitation and emission, the derivation is:

$$\left(P^{-1}-\frac{1}{3}\right)=\left(P_0^{-1}-\frac{1}{3}\right)\left[1+6\frac{\tau R_p(2\cos^2\alpha-1)+R_{op}\cos^2\alpha}{3\cos^2\alpha-1}\right]$$

where P_0 is the limiting polarization, τ the lifetime of the excited state, α the angle

between the emission and absorption oscillators, R_p the rate of rotation about an axis normal to the ring plane, and R_{op} the rate of rotation about an axis contained in the ring plane and at right angles to the absorption oscillator. Also the expression

$$R = \frac{1}{\tau}\left[\frac{P^{-1} - \frac{1}{3}}{P_0^{-1} - \frac{1}{3}} - 1\right] = 6\,\frac{R_p(2\cos^2\alpha - 1) + R_{op}\cos^2\alpha}{3\cos^2\alpha - 1}$$

is only a function of the mobility of the chromophore. As can be seen, it is necessary to know τ and P_0. The change of the excited-state lifetime with temperature is assumed to follow the change in quantum yield q with temperature. On the other hand we have:

$$I \text{ emitted} = I_0(1 - 10^{-\varepsilon cl})q$$

where ε is the molar extinction coefficient of the probe, c its concentration and l the optical depth of solution.

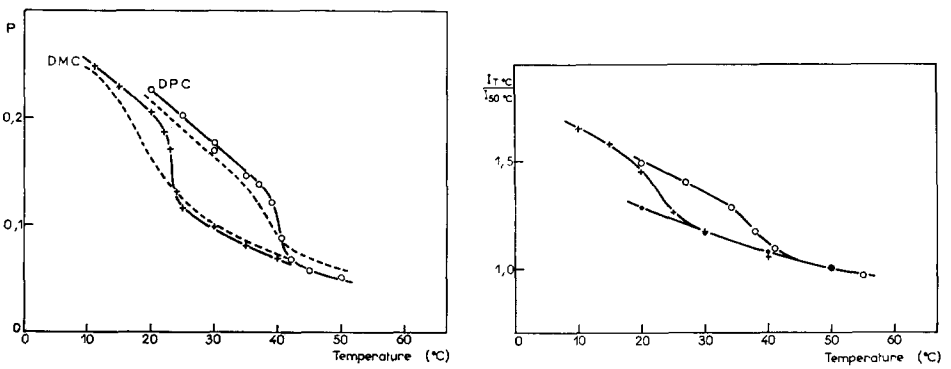


Fig. 5. Effect of temperature on the degree of polarization (P) of the dansyl phosphatidylethanolamine inserted in unsonicated aqueous dispersions of dipalmitoyl- (DPC) (○—○) and dimyristoylphosphatidylcholine (DMC) (+ — +). — — —, results obtained with the corresponding vesicles.

Fig. 6. Change with temperature of the total intensity $I_{/\!/} + 2I_{\perp}$ of the emitted light from vesicles labeled with dansyl phosphatidylethanolamine. ○—○, DPC vesicles; +—+, DMC vesicles; ●—●, EPC vesicles.

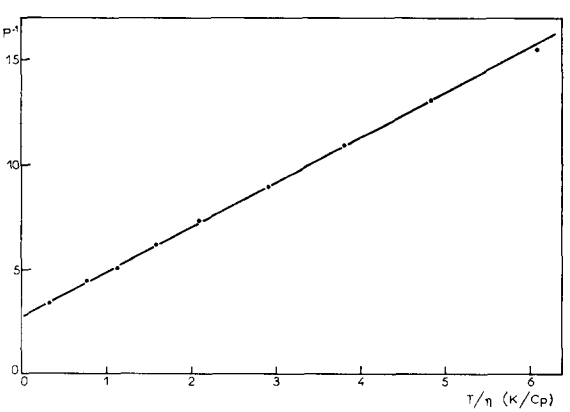


Fig. 7. Perrin's plots of the dansyl phosphatidylethanolamine solubilized in Triton X-100.

Therefore, if the molar extinction coefficient of the probe does not vary during the temperature change, the total intensity emitted $I_{/\!/}+2I_{\perp}$ is proportional to the quantum yield and consequently to the lifetime of the excited state. We have verified that ε of the dansyl phosphatidylethanolamine located in the vesicles is roughly constant over the whole temperature range. The total intensity emitted is plotted as a function of temperature in Fig. 6. The melting of the aliphatic chains of dipalmitoyl- and dimyristoylphosphatidylcholine leads to a decrease of emitted intensity which probably occurs from collisional quenching. To obtain the limiting polarization P_0 of the dansyl phosphatidylethanolamine, we measured the viscosity and P of a solution of Triton X-100 containing the probe. The Perrin's plot (Fig. 7) gives $P_0=0.37$. Since τ of the dansyl phosphatidylethanolamine in egg phosphatidylcholine vesicles is 12.2 ns at 20 °C (ref. 5) and assuming that above the transition temperature τ is the same in the different systems, R can be calculated. As might be expected, the mobility of the probe increases markedly during the phase transition about 14-fold when the temperature changes from 15–50 °C for the dimyristoyl- and dipalmitoyl-phosphatidylcholine systems (Fig. 8).

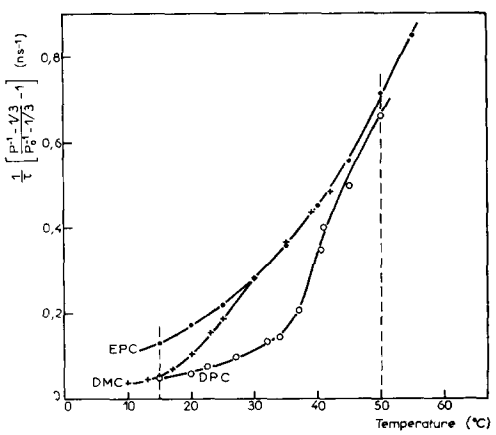


Fig. 8. Variation of the mobility of the dansyl phosphatidylethanolamine located in vesicles of: +—+, dimyristoylphosphatidylcholine (DMC); ○—○, dipalmitoylphosphatidylcholine (DPC) and ●—●, egg phosphatidylcholine (EPC).

CONCLUSION

Dansyl phosphatidylethanolamine and 9-methylanthracene are reliable indicators of order–disorder transitions of the phospholipid aliphatic chains. With 9-methylanthracene, the values of P obtained above the transition temperature are close to those of Shinitzky *et al.*[9], using the 2-methylanthracene as a probe in alkyl trimethylammonium spherical micelles. Thus it may be inferred that above the melting temperature the phospholipid bilayer interior is similar to the hydrocarbon region of these micelles. With the dansyl phosphatidylethanolamine probe, the transition of dipalmitoylphosphatidylcholine is spread over a 34–40 °C temperature range, while this range is only 3 °C with aqueous dispersions. This difference might perhaps be ascribed to the high curvature of the small vesicles, 300 Å diameter, introducing some disorder in the packing of the phospholipid aliphatic chains. In other words,

the phospholipid dispersions are better systems and consequently the two membrane models are not exactly identical as far as the structure near the transition temperature is concerned. The use of 1-mm wide slits is a really efficient procedure allowing the measurement of the degree of polarization of very turbid solutions. This leaves open the possibility of carrying out precise measurements in biological membrane suspensions.

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