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MEROCYANINE 540, A FLUORESCENT PROBE SENSITIVE TO LIPID PACKING

PATRICK WILLIAMSON [a], KAREN MATTOCKS [a] and ROBERT A. SCHLEGEL [b]

[a] *Department of Biology, Amherst College, Amherst, MA 01002 and* [b] *Molecular and Cell Biology Program, The Pennsylvania State University, University Park, PA 16802 (U.S.A.)*

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Binding of the lipophilic probe merocyanine 540 to artificial bilayers was assessed by measuring the enhancement of fluorescence which results when dye enters the hydrophobic environment of the membrane. Titration of a constant amount of dye with increasing amounts of vesicles revealed that much more dye binds to multilamellar and 1000-Å unilamellar vesicles which are in the fluid-phase state than to comparable vesicles which are in the gel-phase state. Incorporation of cholesterol into fluid-phase vesicles at levels of greater than 20 mol% reduced dye binding, whereas cholesterol had no effect at any concentration when incorporated into gel-phase vesicles. Sonicated 200–300-Å unilamellar gel-phase vesicles, which because of their reduced radius of curvature resemble fluid-phase bilayers in their more widely spaced exterior leaflet lipids, bound more dye than 1000-Å unilamellar gel-phase vesicles constructed from the same lipid. These results suggest that merocyanine 540 is able to sense the degree of lipid packing of bilayers and inserts preferentially into bilayers whose lipids are more widely spaced.

Introduction

Microscopic studies designed to reveal the presence and topological distribution of surface components on living cells rely heavily on fluorescent probes of known specificity. Extensive use has been made of fluorophore-labeled macromolecules, such as antibodies, lectins and ligands of specific receptors, to locate and chart the behavior of specific membrane proteins. However, only recently have smaller hydrophobic probes, either naturally fluorescent or conjugated to a fluorophore, begun to yield similar information on the structure and organization of the lipid moiety of the membrane. One particularly interesting member of this latter class of probes is the naturally fluorescent dye, merocyanine 540. When bound to the plasma membrane of living cells, this molecule responds to rapid changes in membrane potential with slight alterations in its absorption and emission spectra [1]. By the use of differential spectroscopic measurements, this property has been exploited to monitor optically action potentials in nerves and axons. Several years ago, however, it was noted that dye binding itself appeared to detect some difference between the surfaces of leukemia cells and their normal counterparts [2,3]. Additional studies have shown that merocyanine 540 recognizes discrete domains in the plasma membranes of leukemia cells [3,4], hematopoietic stem cells [5], immature erythroid cells [6], macrophages (Schlegel, R.A., Phelps, B.M. and Williamson, P., unpublished data) and sperm cells (Schlegel, R.A., Cofer, G.P., Hammerstedt, R., Kozar-

Abbreviations: PC, phosphatidylcholine; DLPC, dilaurylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

sky, K., Friedus, D. and Williamson, P., unpublished data).

An understanding of the nature of these domains necessarily requires information on the factors which influence dye-binding affinity. Fluorescence microscopy has been used to demonstrate that protocols which yield differential binding to the plasma membranes of living cells also yield differential binding to artificial bilayers of differing phase state: fluid-phase bilayers bind merocyanine 540 strongly, while gel-phase membranes do not [4]. The observation that the absorption spectrum of merocyanine 540 in the presence of artificial vesicles changes from an aqueous to a hydrophobic spectrum as the temperature is raised through the phase-transition temperature [7] can be similarly interpreted as indicative of preferential binding to fluid-phase bilayers, as can calorimetric measurements which show that merocyanine 540 incorporated into vesicles induces the appearance of a phase transition at a temperature lower than that exhibited by vesicles of pure phospholipid [8]. However, a direct proof of the tenet inferred from these results has been lacking. We here present, with the use of a dye-titration assay, experiments which directly and quantitatively address the issue of differential dye affinity and which, in addition, provide information on the factors which determine the strength of binding.

Materials and Methods

Lipids were purchased from either Sigma or P.L. Biochemicals and gave similar results. Merocyanine 540, purchased from Eastman Kodak, was dissolved at a concentration of 1 mg/ml in water and stored at 4°C.

Multilamellar vesicles were prepared under N_2 by drying 3 μmol of phospholipid dissolved in chloroform onto the sides of a 15 ml Corex tube. To each tube, heated to 65°C, was added 1 ml of preheated Tris-acetate buffer (0.1 M NaCl, 0.2 mM EDTA, 0.02 M Tris-acetate, pH 8.1). These mixtures were held undisturbed for 30 s, then swirled vigorously for 2 min. Vesicles containing cholesterol were prepared by mixing the sterol dissolved in chloroform with the phospholipid prior to drying. Small (200–300 Å) unilamellar vesicles were prepared by intermittent sonication of a suspension of multilamellar vesicles at 18.5 A with an MSE ultrasonicator fitted with a microprobe tip. Sonication was performed until the suspension was nearly clear (usually 20–30 min). Large (1000–2000 Å) unilamellar vesicles were prepared by the deoxycholate method of Enoch and Strittmatter [9]. Sonicated vesicles, recovered from the supernatant after centrifugation for 10 min in an Eppendorf microfuge, were titrated with deoxycholate to a lipid : detergent ratio of 2. The then turbid suspension was passed over a 2.5 × 30 cm column of Sepharose 4B (200 mesh) in Tris-acetate buffer. Vesicles were taken from the turbid fractions at the exclusion volume and checked by light microscopy for contamination by large aggregates. Vesicle concentrations were determined by phosphorus analysis using the method of Broekhuyse [10], with monobasic sodium phosphate as standard.

Dye-binding assays were carried out by addition of vesicles to dye dissolved at a concentration of 10 μg/ml in Tris-acetate buffer. Fluorescence spectra were taken on a Perkin Elmer Model MPF-3 fluorimeter. Absorption spectra were taken on a Beckman Model 25 ultraviolet-visible spectrophotometer.

Electron microscopy was performed by depositing vesicles on parlodion-coated grids, staining as described by Enoch and Strittmatter [9] and visualizing with a Zeiss EM-9 electron microscope.

Results

Merocyanine 540 is soluble in both aqueous and organic solvents. However, its visible absorption and fluorescence spectra vary with the dielectric constant of the solvent [11]. In particular, in nonaqueous solvents the fluorescence emission spectrum shifts from an aqueous maximum at 570 nm to 590 nm; moreover, this shift is accompanied by a large increase in fluorescence yield [11]. These properties provide a sensitive assay for dye which is bound to bilayers, as illustrated in Fig. 1 where increasing amounts of phospholipid vesicles made from egg phosphatidylcholine (PC) were added to a constant amount of dye. A steady increase in the amount of light emitted occurred concomitantly with a shift in emission maximum to 590 nm.

If merocyanine 540 does bind preferentially to

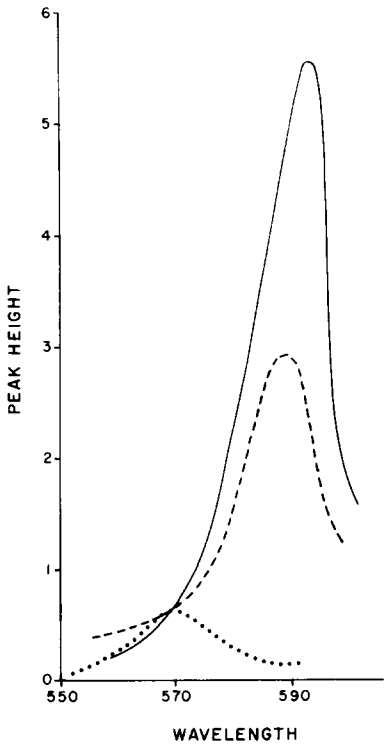

Fig. 1. Merocyanine 540 fluorescence emission spectra in the presence of phospholipid vesicles. Merocyanine 540 was titrated with egg PC multibilayer vesicles as described in Materials and Methods. Excitation at 540 nm was used, and the units of the vertical axis are arbitrary. ·····, No vesicles added; — — —, 0.5 μmol of lipid phosphorus; ———, 0.8 μmol of lipid phosphorus.

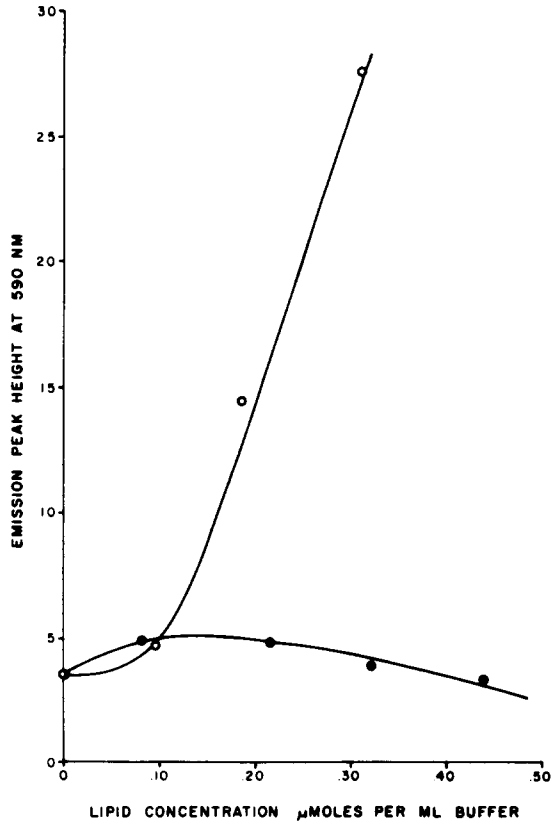

Fig. 2. Phase-state dependence of lipid-dye titration. Merocyanine 540 was titrated as in Fig. 1. Fluorescence emission spectra were taken and intensity at 590 nm plotted as a function of the amount of phospholipid added. O, Fluid-phase DLPC vesicles; ●, DPPC gel-phase vesicles.

fluid-phase bilayers, it should behave differently upon titration with gel versus fluid-phase vesicles. As shown in Fig. 2, addition of vesicles made from dilaurylPC (DLPC), which are fluid at room temperature, resulted in a dramatic increase in dye fluorescence. However, over the same concentration range of total phospholipid, dipalmitoylPC (DPPC) vesicles, which are in the gel-phase state at room temperature, produced a much weaker enhancement. Although this is the result expected if more merocyanine 540 molecules bind to fluid versus gel-phase vesicles, it also could be that equivalent amounts of dye were binding to each type of vesicle, but that dye sequestered into the bilayer of gel-phase vesicles was quenched. However, several considerations argue against this interpretation. Merocyanine 540 fluorescing from gel-phase bilayers exhibits the same fluorescence excitation spectrum as that observed for dye in fluid-phase bilayers (Fig. 3B,D). Bound dye molecules thus appear to reside within equivalent environments in both types of vesicles. However, the amount of dye which displayed this shift was quantitatively less in gel-phase vesicle-dye mixtures than in fluid-phase vesicle-dye mixtures. In Fig. 3A and C, the absorption spectrum of dye molecules was compared for both types of vesicle-dye mixtures. With fluid-phase vesicles, the bulk of the dye molecules have acquired the absorption spectrum characteristic of molecules in a hydrophobic environment. In contrast, at comparable concentrations of gel-phase vesicles, the bulk of the dye still displayed the spectrum characteristic of molecules in an aqueous solvent. Taken to-

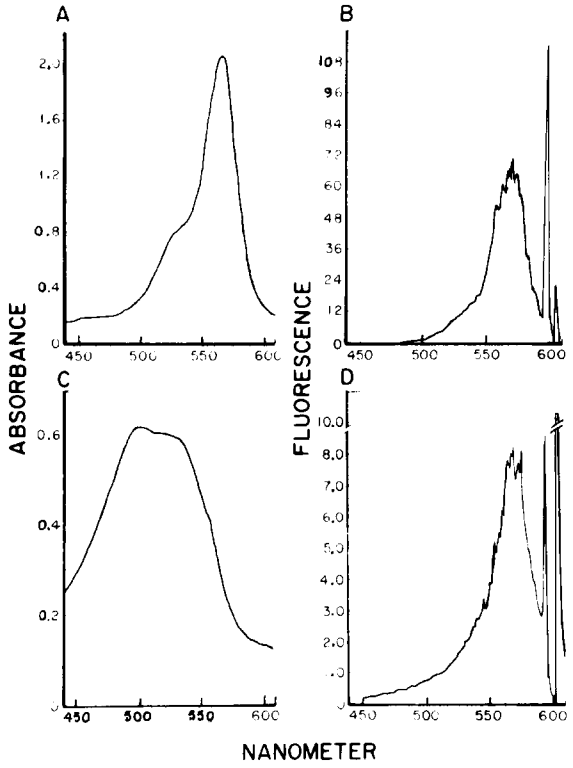

Fig. 3. Absorption and fluorescence excitation spectra for dye/vesicle mixtures. Dye was mixed with equal amounts of gel-phase (DPPC) or fluid-phase (DLPC) vesicles until dye fluorescence at 590 nm was observed from the gel-phase mixtures (approx. 0.8 μmol/ml buffer). Aliquots were then removed and spectra taken. A, absorption spectrum, dye + fluid-phase vesicles; B, fluorescence excitation spectrum (for emission at 595 nm), dye + fluid-phase vesicles; C, absorption spectrum, dye + gel-phase vesicles; D, fluorescence excitation spectrum, dye + gel-phase vesicles.

gether, these data imply that dye molecules in gel-phase membranes are not quenched, but rather that fluid-phase bilayers bind much more dye than do their gel-phase counterparts.

These experiments were performed with multilamellar vesicles. If dye were able to exchange across fluid-phase bilayers much more quickly than across gel-phase bilayers, the disparity in the amount of dye bound might simply be the result of more lipid being accessible to dye in fluid-phase vesicles. While preliminary measurements of fluorescence enhancement as a function of time after addition of vesicles to dye argued against this explanation (data not shown), such a mechanism was ruled out directly by the use of unilamellar vesicles. Using the deoxycholate procedure of Enoch and Strittmatter [9], unilamellar vesicles of 1000–2000 Å diameter were prepared from DLPC. Preparation of DPPC vesicles was effected by a similar procedure, except that all steps of the protocol, including column chromatography on Sepharose 4B, were performed at 55°C, a temperature above the phase-transition temperature of the lipid. Whereas small vesicles (and excess detergent) were included on the column, 1000–2000-Å vesicles appeared in the excluded volume. A typical elution profile for DPPC vesicles is presented in Fig. 4. As judged by light microscopy, the excluded volume fraction was devoid of vesicles larger than 1000–2000 Å or multilamellar products. In the electron microscope, fluid- and gel-phase vesicles prepared by this method were comparable (data not shown). When these unilamellar vesicles were used to titrate MC540, gel-phase vesicles again bound much less dye than did fluid-phase vesicles (Fig. 5), as was the case with multilamellar vesicles. Therefore, differences in dye binding do not depend on the multilamellar character of vesicles, but rather reflect a real difference in dye affinity for vesicles of differing phase state.

Plots of fluorescence intensity versus vesicle concentration were often characterized by a tran-

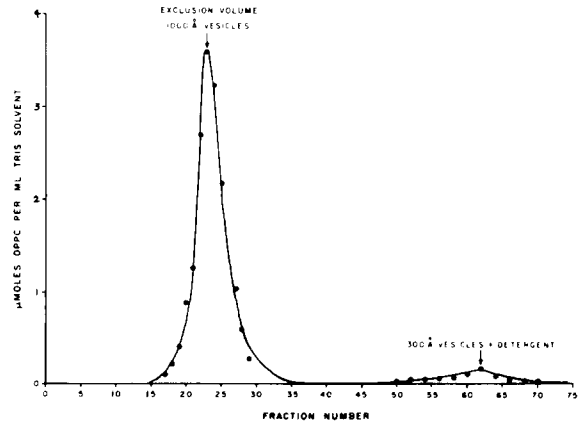

Fig. 4. Gel exclusion chromatography of gel-phase vesicles. A mixture of DPPC and deoxycholate, prepared as described in Materials and Methods, was chromatographed on Sepharose 4B at 55°C. Fractions were collected and analyzed for phosphorus. The exclusion volume for the column is at fraction 25, as determined by Blue dextran elution.

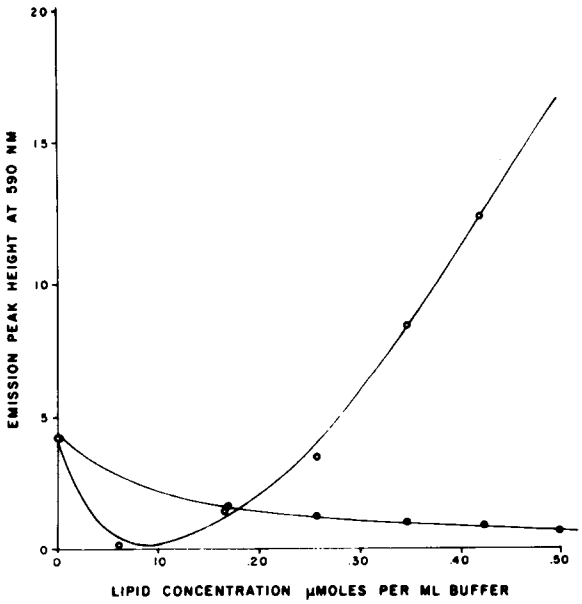

Fig. 5. Titration of merocyanine 540 with unilamellar vesicles. Large unilamellar DPPC and DLPC vesicles, prepared and purified on Sepharose 4B columns, were used to titrate merocyanine 540. O, DLPC; ●, DPPC.

sient decrease in fluorescence intensity as more vesicles were added (see, for instance, Fig. 5). Inspection of excitation and emission spectra taken at the nadir of these plots revealed a blue shift to 520 nm in excitation maxima and a prominent red shift to 620 nm in emission peaks, thus lowering the intensity recorded at 590 nm (data not shown). Such spectral alterations are characteristic of the formation of dye dimers [12]. In the case of gel-phase vesicles, this effect can actually dominate the observed titration curves over the same concentrations where the fluorescence from fluid-phase vesicles is increasing.

The phase-state behavior of phospholipid bilayers is altered by the inclusion of cholesterol. Phase diagrams which summarize a diversity of data indicate that below the phase-transition temperature of PC/cholesterol bilayers at least two phases occur, one below 20% cholesterol, and the other above 20% cholesterol [13,14] (phases II and III, respectively, in the terminology of Recktenwald and McConnell [14]). Above the phase-transition temperature, a single phase (phase I) occurs. However, the transition from phase III to phase I occurs at increasingly elevated temperatures as the percentage of cholesterol increases from 20% in phase III. Therefore, we asked what effect cholesterol has on the binding of merocyanine 540 to phospholipid bilayers. Vesicles containing varying amounts of cholesterol were prepared from either DLPC or DPPC and were used to titrate merocyanine 540 (Fig. 6). The presence of cholesterol in gel-phase DPPC vesicles had no effect on dye binding compared to pure phospholipid; the bilayer displayed the same low affinity for merocyanine 540 at molar concentrations of cholesterol as high as 50%. For the case of fluid-phase vesicles, DLPC vesicles containing up to 20% cholesterol retained their high affinity for the dye. At higher concentrations of cholesterol, however, the affinity of DLPC vesicles for merocyanine

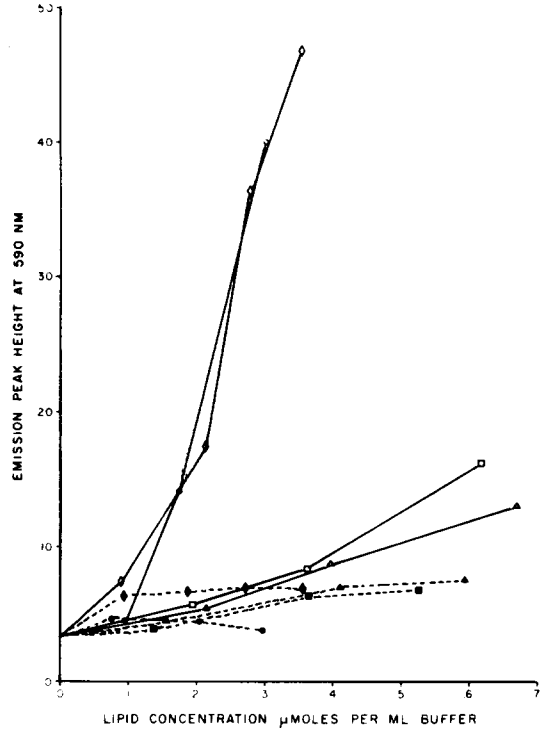

Fig. 6. Effect of cholesterol on merocyanine 540 binding. Cholesterol-containing multibilayer vesicles were prepared as described in Materials and Methods and used to titrate merocyanine 540. Solid lines, open symbols, are titrations with DLPC-containing vesicles; dashed lines, closed symbols, are titrations with DPPC-containing vesicles. Circles, No cholesterol; diamonds, 20% cholesterol; squares, 33% cholesterol; triangles, 50% cholesterol (% = molar proportions).

540 was abruptly suppressed. It would, therefore, appear that merocyanine 540 displays only a very low affinity for phase II and III bilayers and that the inclusion in fluid-phase bilayers of amounts of cholesterol sufficient to induce phase III effectively suppresses the dye binding.

Although the difference between the phase states of a lipid is generally thought of in terms of a difference in fluidity, the high-cholesterol phase III is actually relative fluid [15] even though below the phase-transition temperature. Since merocyanine 540 does not bind this phase (Fig. 6), it is unlikely that dye binding is sensitive to bilayer viscosity per se. Rather, the ability of cholesterol to bring about a condensation of fluid-phase PC molecules [16] suggests that differences in molecular packing at the bilayer surface might be responsible for the difference in affinity of fluid- and gel-phase bilayers for merocyanine 540. To test this possibility, we examined dye binding to sonicated, small unilamellar vesicles, since their very high surface curvature results in outer-leaflet headgroup separation, even in gel-phase vesicles [17]. As Fig. 7 demonstrates, when DPPC vesicles of this type were used to titrate merocyanine 540,

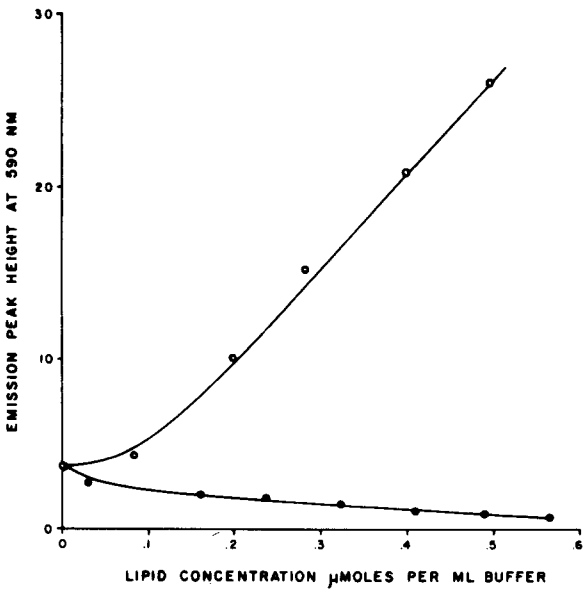

Fig. 7. Comparison of merocyanine 540 binding by large and small unilamellar gel-phase vesicles. Unilamellar DPPC vesicles were used to titrate merocyanine 540. ○, Sonicated 200–300-Å vesicles; ●, 1000-Å vesicles.

they bound dye much more readily than did their low-curvature counterparts, even though 10°C below their (somewhat reduced) phase-transition temperature. These data support the concept that merocyanine 540 binding is influenced by the degree of lipid packing in the bilayer. Binding is weak when the lipids are closely spaced and stronger when they are more widely spaced.

Discussion

Interest in defining the specificity of merocyanine 540 binding stems from the ability of the dye to visualize membrane domains on a variety of cells [3–6] (Schlegel and co-workers, unpublished data). In the course of these microscopic studies we observed visually that merocyanine 540 appeared to bind much more strongly to fluid-phase vesicles than to gel-phase vesicles [4]. The results presented here provide a more quantitative confirmation of this behavior. Monitored by the increase in fluorescence intensity that occurs upon sequestration of dye into membrane, fluid-phase vesicles, in effective dye excess, bind much more merocyanine 540 than do their gel-phase counterparts. This conclusion provides an explanation for a number of observations which have been made with this probe. Lelkes and Miller [7] have shown that as merocyanine 540/DPPC vesicle mixtures pass through the phase-transition temperature of the lipid, the absorbance spectrum of merocyanine 540 becomes progressively more like that of dye in hydrophobic solvents. This is indeed what would be predicted if the number of dye molecules bound to the lipid bilayer increased as it became more fluid, just as was shown to occur in Fig. 3. These same investigators have observed the appearance of an endothermic transition about 4°C below the normal transition temperature of pure DPPC and merocyanine 540 [8]. As the fraction of dye mixed with the vesicles is increased, the fraction of membrane which undergoes this lowered transition also increases. This behavior is consistent with thermodynamic considerations which predict that molecules which interact preferentially with fluid-phase components should lower their phase-transition temperature [18]. In addition, the fact that no change was observed in the phase-transition temperature of the gel-phase lipid which remained

after completion of the lower temperature transition, even at dye-lipid ratios as high as about 1 : 2, suggests that within the limits of detection all of the dye had preferentially associated with the more disordered lipid.

As bilayers traverse their phase-transition temperature, a variety of changes in their properties take place. The viscosity of the bilayer decreases, the membrane expands laterally and shrinks transversely, and fatty acid side-chain motion increases, while the long-range ordering and semicrystalline close packing of headgroups disappears. A loosening of lipid packing near the hydrophilic headgroups, similar to that observed at the phase transition, occurs in sonicated unilamellar vesicles because of the short radius of curvature of the outer leaflet, even though side chains largely remain in a *trans* configuration [17]. The fact that merocyanine 540 binds more strongly to these vesicles than to corresponding larger unilamellar vesicles therefore implicates lipid packing as the primary determinant of merocyanine 540 bilayer affinity.

Although inclusion of greater than 20% cholesterol in gel-phase vesicles decreases membrane microviscosity, merocyanine 540 is still unable to bind. Apparently the lesser area occupied by individual neighboring phospholipid molecules in these PC/cholesterol mixtures, relative to fluid-phase vesicles, effectively prevents dye binding. Thus binding to fluid-phase vesicles is also suppressed at cholesterol concentrations which bring about this rearrangement. In terms of the PC/cholesterol diagram of Recktenwald and McConnell [14] phase III does not bind merocyanine 540, consistent with sensitivity of the dye to lipid packing rather than to fluidity.

The implications of the current study are twofold. First, these experiments provide insights into the nature of the plasma membrane domains which merocyanine 540 detects on a variety of cells, suggesting that relatively loose packing in the proximity of the headgroups of the lipids in these regions is responsible for binding. What remains is to determine the physiological roles of these domains and to establish the molecular basis for the apparent loose packing by identifying biochemically the lipids which comprise these domains. Second, this study emphasizes the importance of developing other probes which are specific for other features of membrane lipid structure or organization. A panel of these probes would be extremely useful in revealing the organization of heterogeneous membrane lipid bilayers as well as the compositional basis for this heterogeneity.

Acknowledgements

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