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Lateral Diffusion of M-13 Coat Protein in Mixtures of Phosphatidylcholine and Cholesterol†

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ABSTRACT: The translational diffusion of fluorescent-labeled M-13 phage coat protein (FITC-M-13), an integral membrane protein, has been measured in mixtures of phosphatidylcholines and cholesterol, using a pattern photobleaching technique. At temperatures below the chain-melting transition temperature of dimyristoylphosphatidylcholine (DMPC) (23.8 °C), the lateral diffusion coefficient of M-13 shows a marked increase when the cholesterol concentration is increased above 20 mol %. A similar marked increase in the lateral diffusion coefficient of a fluorescent phospholipid is also observed. At temperatures above the chain-melting transition temperature of DMPC, a minimum is observed in the lateral diffusion coefficient of FITC-M-13 for cholesterol concentrations in the vicinity of 25 mol %. This minumum in the diffusion coefficient of FITC-M-13 is also observed at 25 mol % cholesterol in egg phosphatidylcholine at 15 °C. No such minimum is observed for the lateral diffusion coefficient of the fluorescent lipid. The lateral diffusion coefficient of FITC-M-13 is large ($>10^{-9}$ cm^2/s) at all cholesterol concentrations for temperatures above the chain-melting transition temperature of the phosphatidylcholine. Several other proteins contain hydrophobic regions similar to that of the M-13 coat protein. We anticipate that a number of proteins of this type will show similar diffusional behavior, in particular exhibiting rapid diffusion throughout a wide range of lipid composition.

In recent work the lateral diffusion coefficient of the M-13 phage coat protein in dimyristoylphosphatidylcholine (DMPC)[1] was measured by using a technique involving fluorescence redistribution after pattern photobleaching (Smith & McConnell, 1978; Smith et al., 1979a). In other recent work we have observed a surprising effect of cholesterol concentration on the lateral diffusion coefficient of fluorescent phospholipid in binary mixtures of phosphatidylcholines and cholesterol at temperatures below the chain-melting transition temperature of the phosphatidylcholine; increasing cholesterol concentration in the bilayer above ~20 mol % leads to a sharp, order-of-magnitude increase in the diffusion coefficient of a fluorescent phospholipid (Rubenstein et al., 1979). This unusual effect has now been accounted for in terms of a remarkable lipid bilayer structure in which stripes of pure solid phosphatidylcholine alternate in a regular way with stripes of fluid lipid, the fluid lipid containing 20 mol % cholesterol (Copeland & McConnell, 1980; Owicki & McConnell, 1980; Rubenstein et al., 1980). The several studies described above prompted the present work. Thus, it was anticipated that a comparable abrupt change in the lateral diffusion of this intrinsic membrane protein would be observed when cholesterol concentration is increased above 20 mol % in binary mixtures of DMPC and cholesterol at temperatures below the chain-melting transition temperature of the phospholipid (23.8 °C). This expectation is confirmed here.

A second motivation for the present study stems from the paucity of biophysical studies of the effects of cholesterol on the properties of integral membrane proteins. [For two references see Kleeman & McConnell (1976) and Warren et al. (1975).] In contrast, extensive studies have been made of the biophysical properties of phosphatidylcholine–cholesterol mixtures. [For a review, see Demel & De Kruyff (1976).]

A recent report on the lateral diffusion of an amphipathic apolipoprotein bound to phosphatidylcholine and phosphatidylcholine–cholesterol bilayers has been made by Vaz et al. (1979). Their results are consistent with those presented here in that rapid diffusion of the apolipoprotein is reported.

Materials and Methods

Materials. Dimyristoylphosphatidylcholine (DMPC) was obtained from Sigma and used without further purification. A stock solution in methanol was stored at 4 °C. Egg phosphatidylcholine was isolated as described by Ross & McConnell (1977). A stock solution in ethanol was stored at −20 °C. Concentrations of the phospholipid stock solutions were determined by phosphate assay (McClare, 1971). Cholesterol was recrystallized twice from ethanol and stored in ethanol and under argon at −20 °C. The concentration of

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[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; FITC, fluorescein isothiocyanate; FITC-M-13, M-13 phage coat protein conjugated with FITC; *D*, lateral diffusion coefficient; NBD egg PE, fluorescent-labeled egg phosphatidylethanolamine; NBD-DMPE, a fluorescent-labeled dimyristoylphosphatidylethanolamine, *N*-(4-nitrobenzo-2-oxa-1,3-diazolyl)-L-α-dimyristoylphosphatidylethanolamine.

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the stock solution was determined by gravimetric analysis. *N*-(4-Nitrobenzo-2-oxa-1,3-diazolyl)-L-α-dimyristoylphosphatidylethanolamine (NBD-DMPE) was obtained from Avanti Biochemicals by special order. Stock solutions (0.01 mM in methanol) were kept at 4 °C. M-13 bacteriophage (10 mg/mL in 0.01 M Tris-HCl, 0.1 mM EDTA) was the generous gift of William Wickner.

M-13 Coat Protein. M-13 coat protein was purified by the method of Knippers & Hoffman-Berling (1966). The lyophilized protein was solubilized in 1% sodium cholate (Sigma) and 20 mM carbonate/bicarbonate buffer, pH 8.5, by stirring 4 h at room temperature and removing undissolved protein by centrifugation at 35000*g* for 30 min at room temperature. Final protein concentration was 1.4 mg/mL as determined by UV absorption at 280 nm (Nozaki et al., 1976). This solution was stored at 4 °C in the presence of 0.1% sodium azide. FITC-conjugated coat protein was prepared as previously described (Smith et al., 1979a).

Reconstitution. Fluorescent-labeled coat protein was incorporated into model membranes for diffusion measurements by three different methods. The first two methods involve acetone-precipitating the protein out of detergent solution, suspending it in MeOH/H_2O (2:1), adding this solution to appropriate amounts of lipids, rotoevaporating to a thin film, and then either preparing multibilayer samples (method 1) or preparing multilamellar liposomes (method 2). In the third method, the protein is reconstituted into small vesicles by the cholate dilution method (Wickner, 1977), lyophilized, mixed with added organic solvent, rotoevaporated to a thin film, and made into liposomes. Estimated uncertainty in cholesterol concentration is ±1 mol % cholesterol for all samples. The details are as follows.

Methods 1 and 2. Suspensions of FITC-M-13 in MeOH–H_2O (2:1) were prepared by coprecipitation of the protein with bicarbonate buffer. To 0.2 mL of 0.06 mM FITC-M-13 in 1% sodium cholate and 20 mM sodium bicarbonate, pH 8.5, was added 3 mL of acetone. This mixture was cooled in a dry ice–2-propanol bath for approximately 15 min and centrifuged at 4 °C and 1200*g* for 10 min. The pellet was resuspended in 2 mL of acetone, and 0.1 mL of 20 mM $NaHCO_3$, pH 9.0, was added. This solution was cooled and centrifuged as before, and the precipitate was resuspended in 0.1 mL of MeOH/H_2O (2:1). To 0.7 μmol of mixtures of phospholipid and cholesterol in ethanol was added 1 nmol of the FITC-M-13 in suspension. The solvent was then rotoevaporated to a thin film. Residual cholate was less than 0.1 mol % relative to lipid as estimated by using [^{3}H] cholate (New England Nuclear).

One-tenth milliliter of chloroform was added to the lipid–protein film. Oriented multibilayers (method 1) were prepared from this chloroform solution as previously described (Rubenstein et al., 1979; Smith et al., 1979a). For the preparation of multilamellar liposomes using the suspended protein (method 2), the chloroform solution was again rotoevaporated to a thin film, 1 mL of phosphate-buffered saline was added, and the mixture was kept at 40 °C for 15 min and then vortexed vigorously for 15 s to form the liposomal suspension.

Method 3. Small vesicles of DMPC and FITC-conjugated coat protein in water were prepared as previously described (Smith et al., 1979a), except that 100 mM Tris buffer, pH 7.3, was used in place of 100 mM phosphate buffer. The phospholipid concentration of the vesicular suspension was determined by phosphate assay to be 5.7 mM (McClare, 1971), sodium azide was added to 0.1%, and the suspension was stored at 4 °C. Aliquots (0.1 mL) of vesicles were lyophilized, appropriate amounts of 10 mM cholesterol in ethanol were added to give the desired mole ratios of phospholipid to cholesterol, 0.1 mL of $HCCl_3$/MeOH (2:1) was added, and the mixture was rotoevaporated to a thin film. One millilter of phosphate-buffered saline was added, and the flask was kept at 40 °C for 15 min and then vortexed vigorously for 15 s to form the liposomal suspension.

Multilamellar liposomes prepared by either method 2 or method 3 were washed before use to remove background fluorescence. To 0.1 mL of the liposomal suspension was added 0.9 mL of phosphate-buffered saline. This was centrifuged at 2500*g* for 2 min to pellet the liposomes. The supernatant was removed and the pellet resuspended in 0.1 mL of phosphate-buffered saline.

Liposomes Containing NBD-DMPE. Liposomes containing NBD-DMPE were prepared as follows.. Aliquots of the DMPC, cholesterol, and NBD-DMPE stock solutions to give of the order of 1-μmol total lipid, the desired mole ratio of phospholipid to cholesterol, and 0.02 mol % NBD-DMPE were combined in a small flask and rotoevaporated to a thin film, and liposomes were made as described above for the coat protein containing liposomes. Occasionally erratic and poorly reproducible results were obtained for these liposomes, particularly in the 0–20 mol % cholesterol and below 23 °C temperature-composition region. In these cases, fresh samples were mixed, and one additional rotoevaporation to a thin film from pure chloroform was included prior to the liposome preparation (Rubenstein et al., 1980). These samples gave consistent and reproducible results.

Diffusion Measurements. Diffusion measurements were made by the fluorescence recovery after pattern photobleaching (FRAPP) technique by using two different methods of measuring the recovery. All measurements on the multibilayer samples were carried out as previously described (Smith et al., 1979a; Smith & McConnell, 1978) by photomicrography of the striped pattern followed by densitometry of the photomicrographs. Measurements on liposomes were carried out as described in Smith et al. (1979b), except that some further refinements of the apparatus have been made. A brief description of the present apparatus follows.

The beam from an argon ion laser (Spectra Physics 164-05) is divided into two independent parts by using two 10% beam splitters and two mirrors to split and recombine the beam. The weaker (observation) beam constitutes ~1% of the initial laser power and the stronger (bleach) beam ~80%. Both beams are separately controlled by using electronic shutters (Vincent Associates), and attenuating filters may be placed in the observation beam to attain the desired intensity without diminishing the intensity of the bleach beam. The recombined beam is expanded by using a pair of lenses to 1–2-cm diameter and reflected into the rear of the microscope (Zeiss Photomicroscope III, equipped with epifluorescence) from a mirror mounted on the microscope. It then passes through a Ronchi ruling which is imaged onto the fluorescent sample to give a striped pattern. All measurements reported here were made with a 40× (numerical aperture = 0.65) planachromat objective focusing a 8.7-μm period striped pattern onto the samples.

The fluorescence was measured by using a cooled photomultiplier tube (RCA C31034-02). The anode current was amplified and the signal displayed on a storage oscilloscope and also sent to a computer (Digital PDP8/E) for storage and analysis. For measurements of a total time duration greater than 7 s, the observation beam was chopped to minimize bleaching of the sample. Typically, 100 data points of 25-ms length were taken. Under these conditions, no measurable

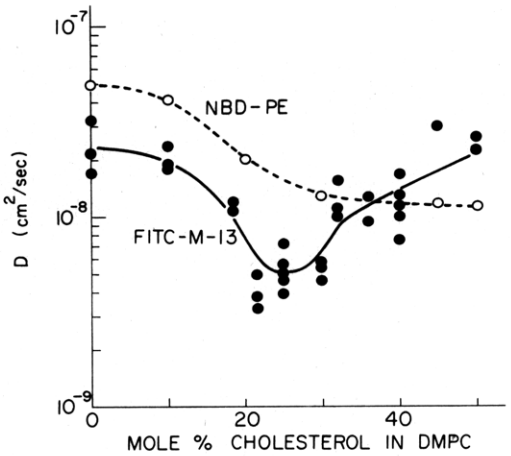

FIGURE 1: Diffusion of FITC-M-13 (●) and NBD egg PE (○) in multibilayers of DMPC and cholesterol at 26 ± 0.5 °C. The lipid diffusion data are taken from Rubenstein et al. (1979).

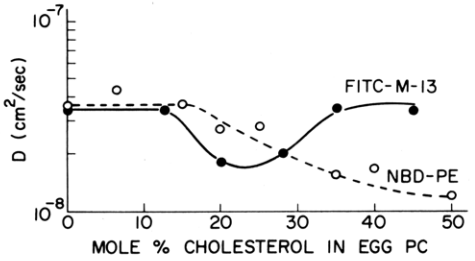

FIGURE 2: Diffusion of FITC-M-13 (●) and NBD egg PE (○) in multibilayers of egg phosphatidylcholine and cholesterol at 15 ± 0.5 °C. The lipid diffusion data are taken from Rubenstein et al. (1979). Each FITC-M-13 point represents a single measurement.

bleaching by the observation beam was detected.

Data were analyzed by computer least-squares fit to a single exponential to give the time constants for recovery. Diffusion constants were calculated by using the relation

$$D = 1/a^2\tau \qquad (1)$$

where a is the spatial frequency of the pattern and τ is the time constant for exponential recovery (Smith & McConnell, 1978; Smith et al., 1979b).

Results

Figure 1 gives the measured lateral diffusion coefficients for FITC-M-13 in multibilayers of DMPC and cholesterol at 26 °C, a temperature above the chain-melting transition temperature of DMPC (23.8 °C). The lateral diffusion coefficient of FITC-M-13 in the multibilayer samples shows a pronounced broad minimum for cholesterol concentrations in the 20–30 mol % range. At cholesterol concentrations greater than 30–40 mol %, the protein diffuses more rapidly than the lipid at that composition and temperature.

Figure 2 gives the measured lateral diffusion coefficients for FITC-M-13 in multibilayers of egg phosphatidylcholine and cholesterol at a temperature of 15 °C, where egg phosphatidylcholine is "fluid". Again a pronounced minimum is seen in the 20–30 mol % cholesterol range, and again the protein diffuses more rapidly than the lipid at high cholesterol concentrations.

Figure 3 shows the measured lateral diffusion coefficients for FITC-M-13 in multibilayers of DMPC and cholesterol at a temperature of 19 °C, below the chain-melting transition temperature of pure DMPC. Fluorescence in these samples was uniform even below 23.8 °C, and the redistribution of fluorescence after-pattern photobleaching was always complete.

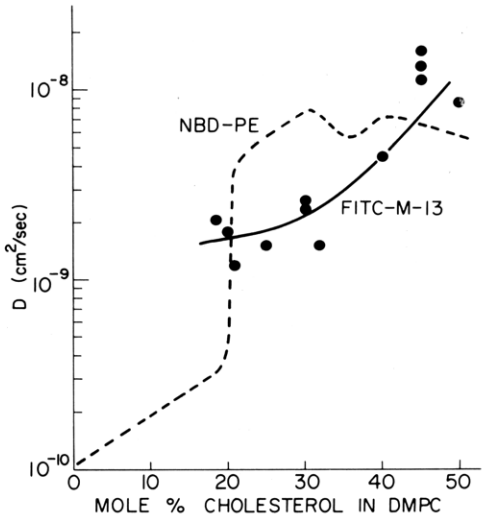

FIGURE 3: Diffusion of FITC-M-13 (●) and NBD egg PE (---) in multibilayers of DMPC and cholesterol at 19 ± 0.5 °C. The lipid diffusion data are taken from Rubenstein et al. (1979). No data are shown for FITC-M-13 diffusion between 0 and 20 mol % cholesterol because variable results have been obtained in this region (see text).

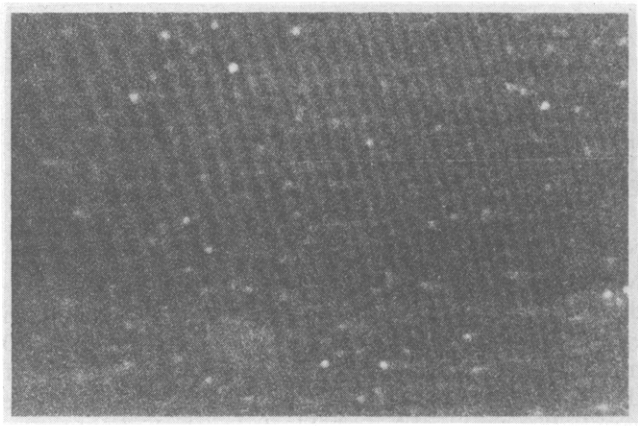

FIGURE 4: Representative "bands" of FITC-M-13 often seen in cholesterol/DMPC samples in the range 15–45 mol % cholesterol. Photograph is 500 × 800 μm.

Data are shown only for the 20–50 mol % cholesterol region as diffusion measurements in the 0–20%, 19 °C composition–temperature region gave highly variable results in these multibilayers.

For cholesterol concentrations between 15 and 45 mol %, we have often observed fluorescent "bands" in the multibilayer samples due to concentrated regions of FITC-M-13. An example is shown in Figure 4 These bands often appear to radiate in concentric ellipsoids from a single point, and Figure 4 shows only a limited region of this pattern. This phenomenon may arise from pattern precipitations similar to those discussed, for example, by Ross (1974). Regions containing putative precipitates were avoided whenever possible in making diffusion measurements; these precipitates themselves did not appear to diffuse or to inhibit the rate of diffusion of unprecipitated FITC-M-13 in the same region. These bands are not due to spatial noise in the exciting radiation because (i) when viewed through the microscope eyepiece, the banded pattern moves when the sample is translated, which is not the case for a spatial noise pattern; (ii) the same bands are also observed when the excitation source is a mercury arc lamp, yielding incoherent radiation; (iii) mechanical manipulation of the sample causes the bands to break up.

Figure 5 shows the measured diffusion coefficients for coat protein in liposomes prepared by method 3, as well as for the

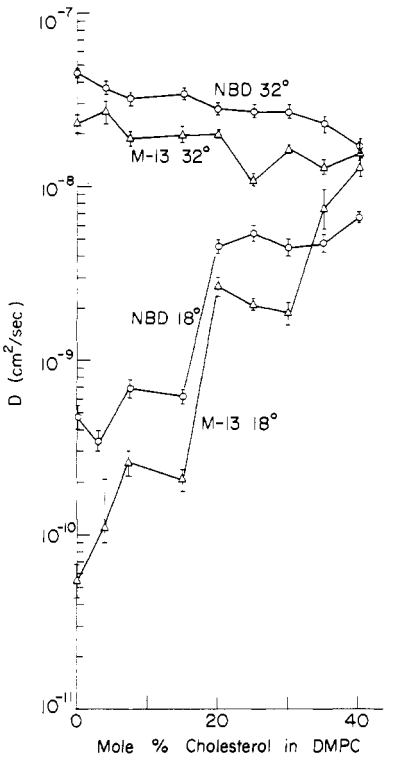

FIGURE 5: Diffusion of FITC-M-13 and NBD-DMPE in multilamellar liposomes of DMPC and cholesterol at both 18 ± 0.5 °C and 32 ± 0.5 °C. An average of five measurements were made for each point. Error bars represent the standard error of the measurements.

NBD-DMPE containing liposomes, at both 18 and 32 °C. Measurements on these liposomes gave consistent and reproducible results throughout the temperature–composition region examined. Again a minimum is observed between 20 and 30 mol % cholesterol above the phase-transition temperature of the phospholipid. This minimum is shallow compared to those seen in Figures 1 and 2, probably due to the higher temperature (32 °C) employed for the data in Figure 5. Once again, the FITC-M-13 protein diffuses at a rate greater than or equal to that of NBD-PE at high cholesterol concentrations. Figure 6 shows a typical recovery curve together with a theoretical fit to a single exponential. As may be seen, signal-to-noise is high and the fit to a single exponential quite good. In all measurements reported here a good fit to a single exponential was obtained.

The diffusion data for the liposomes prepared by method 2 are not shown here since they do not differ substantively from the other data given, except that there is greater scatter and lack of reproducibility, particularly in the low temperature cholesterol region.

It is worth noting that in all the coat protein samples prepared by any of the three methods, the fluorescence intensity diminished significantly above 30 mol % cholesterol. In these protein-containing liposomes above 30 mol % cholesterol, one could often observe microscopic nonuniform and punctate fluorescence. This did not, however, change the amplitude of fluorescence recovery in these samples, indicating that the punctate fluorescence did not contribute to the measured diffusive recovery.

A number of phenomena other than relaxation of the photobleached striped pattern can lead to apparent recovery curves when measurements are made by using the photomultiplier tube (i.e., for all measurements on liposomes). Some of these are (i) fluorescent material in solution diffusing into the observation area after the bleach pulse, (ii) spatial inhomogeneities in the laser beam superimposed on the striped pattern, and (iii) the polarized light of the laser beam interacting with anisotropically distributed fluorescent molecules to give a spatial bleach pattern (Smith et al., 1980). All of these phenomena will lead to an apparent recovery curve even in the absence of a striped pattern.

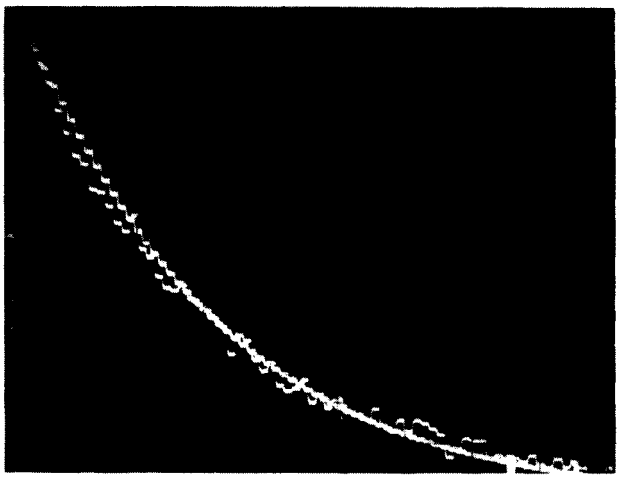

FIGURE 6: A typical fluorescence recovery curve for FITC-M-13 in liposomes of DMPC and cholesterol. The smooth curve is the computer least-squares fit to a single exponential, the rougher curve the measured data points. This sample is 30 mol % cholesterol at 18 °C. The time constant for recovery was 11.8 s, giving a diffusion coefficient $D = 1.5 \times 10^{-9}$ cm^2/s.

A fourth and distinct type of behavior has been observed in some of the coat protein containing liposomes. This is an increase in the fluorescence intensity of a liposome after bleaching (no grid present), occurring on a time scale much longer than the diffusion time scale. Repeated bleaching of the same liposome causes an increase in the relative amplitudes of recovery. This behavior has not been thoroughly investigated, but we tentatively believe that it is due to slow dissociation of clusters of the M-13 coat protein in which the coat protein is less readily destroyed by the bleach beam and less fluorescent.

Discussion

From the present study we reach a number of conclusions:

(i) The marked enhancement in the lateral diffusion of the fluorescent lipid NBD-PE reported previously when cholesterol concentration is increased above 20 mol % in a phosphatidylcholine below its chain-melting transition temperature is also observed in the present work for liposomes. [In a previous work this jump in diffusion was observed in coplanar phospholipid lipid multibilayers (Rubenstein et al., 1979).] As expected, a similar increase in the lateral diffusion coefficient of the M-13 phage coat protein is observed when cholesterol concentration is increased above 20 mol % in liposomes, below the chain-melting transition temperature. This result is in accord with our previous interpretation of the structure of DMPC–cholesterol mixtures for cholesterol concentrations less than 20 mol % and temperatures less than the chain-melting transition temperature. According to this interpretation, the lipid bilayer consists of alternating bands of "solid" DMPC and "fluid" lipid (a binary mixture of DMPC and cholesterol, cholesterol being present at 20 mol %) (Copeland & McConnell, 1980; Owicki & McConnell, 1980; Rubenstein et al., 1980). In the case of the M-13 coat protein, the bands of solid DMPC act as barriers for the macroscopic diffusion of M-13, probably for two reasons. First, the diffusion coefficient of monomeric M-13 in solid DMPC is doubtless

low. Second, the solubility of monomeric M-13 in solid DMPC is also low (Smith et al., 1979a). [For a detailed discussion, see Owicki & McConnell (1980).] At 20 mol % cholesterol, the bands of solid DMPC approach infinite separation, i.e., they disappear so that rapid, long-range diffusion of both lipids and M-13 is possible.

(ii) As can be seen in Figure 1, the lateral diffusion coefficients of FITC-M-13 in coplanar multibilayers consisting of binary mixtures of DMPC and cholesterol at 26 °C show a pronounced minimum in the region between 20 and 30 mol % cholesterol. A similar minimum is seen in Figure 2 for coplanar multibilayers of cholesterol and egg phosphatidylcholine at a temperature (15 °C) where egg phosphatidylcholine is "fluid". This minimum is also present in the diffusion coefficients for FITC-M-13 in liposomes as seen in Figure 5 at 25 mol % cholesterol and at a higher temperature (32 °C). These nonmonotonic variations of diffusion coefficients are not entirely unexpected since the paramagnetic resonance spectra of phospholipid spin labels incorporated in binary mixtures of cholesterol and DMPC show large, nonmonotonic variations in line shape with cholesterol concentration in the 20–40 mol % range, at temperatures both above and below the chain-melting transition temperature of DMPC (Rubenstein et al., 1980). It is possible that the minimum is due to variations in lipid order and consequent variations in lipid–protein interactions. In particular, the spin-label spectra show a minimum in head-group mobility for cholesterol concentrations of the order of 20 mol % in DMPC at 33 °C. Other evidence for inhibition of head-group motion in the 20–35 mol % cholesterol concentration range and at higher temperatures has been obtained by using deuterium and phosphorous nuclear magnetic resonance (Oldfield et al., 1978; Shepherd & Büldt, 1979). It is also possible that the minimum in the diffusion coefficients of FITC-M-13 at 15 mol % cholesterol may be directly related to protein–protein associations, but such protein–protein associations are clearly related to variations in lipid composition.

(iii) The diffusion of the M-13 phage coat protein above the chain-melting transition temperature is rapid throughout the entire range of cholesterol concentrations (See Figures 1, 2, and 5). This protein contains a segment of 19 hydrophobic amino acids which probably constitute the main membrane-binding portion (Chamberlain et al., 1978; Hagen et al., 1978; Wickner, 1977). Inasmuch as the diffusion of molecules with molecular weights $\lesssim 100\,000$ in aqueous solutions is generally much faster than the diffusion of membrane-bound molecules, the rate of diffusion of the protein must be determined primarily by the rate of diffusion of the membrane-binding segment. Since many other membrane proteins also have only short segments of about 20 hydrophobic amino acids in the membrane (e.g., glycophorin, Segrest & Kohn, 1974; Marchesi et al., 1976; H-2, Cunningham, 1977; HLA, Springer & Strominger, 1976; Walsh & Crumpton, 1977; and VSV G protein, Lodish & Rothman, 1979), it is probable that these proteins will also exhibit rapid diffusion comparable to that measured for the M-13 coat protein. This rapid motion of the M-13 coat protein is observed in membranes over a broad range of cholesterol concentrations, suggesting that even in membranes of widely varying composition and physical properties, other proteins containing similar hydrophobic regions will also diffuse rapidly, provided a solid lipid phase is not present and provided protein–protein interactions do not inhibit diffusion.

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