(residue 37) appears to be exposed. The present charge-transfer data indicate that one tryptophan residue and one tyrosine residue are available to interact with *N*-methylnicotinamide chloride, suggesting that these residues are exposed to the solvent. X-ray crystallographic analysis may be needed to resolve the differences between chemical and charge-transfer evidence.

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Lateral Diffusion of M-13 Coat Protein in Model Membranes†

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ABSTRACT: A fluorescent derivative of the M-13 phage coat protein (molecular weight 5260) was reconstituted into oriented multilayers and giant liposomes of dimyristoylphosphatidylcholine. The rate of lateral diffusion of the labeled protein in the fluid phase was measured as a function of temperature and found to be comparable to that of lipid probes. The protein was found to have a nonuniform lateral distribution in the solid phase of both types of model membranes. Cardiolipin (0.5 mol %) included in the multibilayers did not have any substantial effect upon the rate of diffusion.

The lateral motion of membrane components is an important factor in cellular immune response (Schlessinger et al., 1976a; Edelman, 1976). In recent years, several techniques have been developed for the measurement of rates of lateral motion of cell surface components (Devaux & McConnell, 1973; Scandella et al., 1972; Sheats & McConnell, 1978; Smith & McConnell, 1978; Kuo & Wade, 1979; Axelrod et al., 1976). Lateral diffusion coefficients have been reported ranging from 10^{-7} cm^2/s to less than 10^{-11} cm^2/s (Devaux & McConnell, 1973; Edidin & Fambrough, 1973; Frye & Edidin, 1970; Peters et al., 1974; Poo & Cone, 1974; Scandella et al., 1972; Schlessinger et al., 1976b; Sheats & McConnell, 1978; Smith & McConnell, 1978; Wu et al., 1977), with cell surface components sometimes appearing immobile on the time scale of the measurements (Peters et al., 1974; Schlessinger et al., 1976b). The interpretation of some of these results is difficult due to the paucity of knowledge about the physical and dynamical properties of membrane proteins. For such interpretations it is crucial to determine the properties of membrane proteins in simple, well-defined model membranes.

We have used fluorescence redistribution after pattern photobleaching (Smith & McConnell, 1978) to measure the rate of lateral diffusion of a fluorescent derivative of the M-13 phage coat protein, reconstituted into both oriented phospholipid multibilayers and giant multilamellar liposomes. The M-13 phage coat protein is a small hydrophobic protein of molecular weight 5260 (Wickner, 1975). It is easily isolable (Makino et al., 1975; Wickner, 1975), its amino acid sequence is known (Asbeck et al., 1969; Nakashima & Konigsberg, 1974), and its properties have been well characterized by a number of research groups (Chamberlain et al., 1978; Hagen et al., 1978; Knippers & Hoffman-Berling, 1966; Makino et al., 1975; Nozaki et al., 1978; Wickner, 1975; Zwizinski & Wickner, 1977). We have measured the temperature dependence of lateral diffusion of the coat protein in the fluid phase of oriented multibilayers and have studied the effect of the phospholipid phase transition. We have also examined the effect of including cardiolipin in the bilayers since evidence

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indicating an association of cardiolipin with the M-13 phage coat protein has been reported (Chamberlain & Webster, 1976).

Materials and Methods

Materials. M-13 phage coat protein was the generous gift of Dr. William Clark. Sodium cholate (98% pure according to manufacturer's specifications), DMPC[1] (estimated purity >98% by thin-layer chromatography), and FITC were obtained from Sigma Chemical Co. and used without further purification. Bacterial cardiolipin was obtained from Supelco, Inc.

Labeling of Protein. Coat protein, initially in 100 mM Tris and 30 mM deoxycholate, pH 7.0, was exchanged into 30 mM sodium cholate and 50 mM sodium carbonate–bicarbonate buffer, pH 9.0 (buffer A), on a Sephadex G-25 Fine (Pharmacia) column of dimensions 1.0 × 25 cm. Coat protein (50 nmol) was diluted to 1 mL with buffer A, 2.5 μmol of FITC was added, and the solution was stirred at 4 °C for 24 h. This reaction mixture was loaded onto a Sephadex G-25 Fine column (1.0 × 25 cm) preequilibrated with 1% sodium cholate and 20 mM sodium bicarbonate, pH 8.5, and the column was run in this buffer at ~0.2 mL/min. The yellow leading band containing labeled M-13 coat protein was collected, and both the approximate number of fluorescein groups per protein molecule and the concentration of protein were determined spectrophotometrically (Goldman, 1968; Nozaki et al., 1976). The preceding reaction conditions result in a fluorescein to protein mole ratio of 0.5.

Reconstitution. The labeled protein was reconstituted into DMPC vesicles by using a slight modification of Wickner's procedure (Wickner, 1977). To 0.5 mL of the labeled protein solution was added 0.2 mL of DMPC dispersion (40 mg/mL in 100 mM potassium phosphate buffer, pH 7.0 (buffer B), briefly sonicated above 24 °C). This was kept at 23–24 °C for 30 min and then diluted rapidly with 20 mL of buffer B at the same temperature. The solution was maintained at 23–24 °C for 1 h while vesicles formed. The cloudy mixture was centrifuged at 44000*g* for 1 h at 23–24 °C, the supernatant was removed, and the pellet was resuspended in 20 mL of 10 mM potassium phosphate buffer, pH 7.0. It was centrifuged as before, and the pellet was resuspended in 20 mL of glass-distilled water to which sufficient dilute NaOH had been added to raise the pH to between 8 and 10. This suspension was held at 23.8 °C for 1 h and then centrifuged and resuspended in 20 mL of water (pH 8–10) as before. Residual cholate at this point was 0.6 mol % relative to lipid, as determined by ^{3}H-labeled cholate (New England Nuclear). Two further washes lowered the fraction of cholate to 0.2 mol %. This vesicle preparation was lyophilized and stored dry at 4 °C.

Multibilayers. Oriented multibilayers were prepared as previously described (Smith & McConnell, 1978; Sheats & McConnell, 1978; Rubenstein et al., 1979). Chloroform (100 μL per 1–2 μmol of lipid, Burdick & Jackson Laboratories, Inc.) was added to the lyophilized preparation of labeled coat protein and lipid. For some experiments other membrane components (e.g., cardiolipin, additional DMPC) were added at this point. Chloroform solution (100 μL) was applied in small drops to a microscope slide on a heating block held at 50 °C. The drops were applied as uniformly as possible over an area the size of a microscope cover slip (18 × 18 mm). The samples were each allowed to dry for 3–4 min on the heating block and then were placed in a container over distilled water. The container was closed and kept at 40 °C for 24 h. Cover slips were applied to the samples at 50 °C, and the samples were returned to the container and kept at 40 °C for 12 h. They were then kept at room temperature over distilled water until use.

Giant Multilamellar Liposomes. Liposomes were prepared by a variation of the method of Mueller (Antanavage et al., 1978). Chloroform solution (100–200 μL) of labeled coat protein and lipid (see above) was dried by rotoevaporation in a 10-mL pear-shaped flask. Triply distilled water (5 mL) was added, and the flask was kept at 40 °C for 4 days without any agitation and then was gently swirled. The liposomes obtained by this method were predominantly large (20–50 μm), spherical, and multilamellar, as determined by examination under the light microscope.

Diffusion Measurements. Diffusion was measured by using the pattern photobleaching technique as previously described (Smith & McConnell, 1978). The diffusion coefficient for a fluorescent species is determined by measuring the rate of decay of the amplitude of a periodic stripe pattern which has been produced in the sample by intense laser illumination. Diffusion of the fluorescent molecules produces a single exponential decay of the stripe amplitudes. Other types of motion in the sample such as bulk flow of the lipid bilayers can be easily distinguished from diffusion since they produce changes in the positions and shapes of the stripes. Fluid-phase measurements were made in multibilayer membranes by using a stripe period of 85 μm. Only completely oriented regions were used, as determined by their uniformity under Nomarski optics *and* under fluorescence. The durations of the photobleaching pulses were from 1 to 3 s with a power of ~250 mW (488-nm line of the argon ion laser). This gave a decrease of fluorescence intensity of ~70% in the exposed areas. Photomicrographs were taken by using illumination from a mercury arc lamp. Solid-phase measurements were carried out on giant liposomes and utilized a stripe period of 5.3 μm. Measurements on giant liposomes employed a 40× water immersion objective and a microscope slide with a concave well. Liposome suspension (10 μL) was deposited in the well which was then filled with distilled water. Diffusion measurements were made on the top surfaces of giant liposomes which were resting on the slide. Photomicrographs were made with the aid of an image intensifier (Ni-Tec, Inc.). Temperature was directly monitored by using a thermistor immersed in the well.

Results

The multibilayer membranes were not suitable for diffusion measurements below the phospholipid chain melting temperature T_m (23.8 °C for DMPC) due to severe distortion of previously oriented regions and clumping of the fluorescence intensity. Multibilayer samples which had been slowly cooled to below T_m often developed graininess and texture when viewed with Nomarski optics; previously uniform fluorescence often became patchy and mottled (see Figure 1). These changes were reversible, as an increase in temperature to above T_m always restored homogeneity. Diffusion measurements below T_m gave highly variable results, with the lateral diffusion coefficient D ranging from 10^{-9} cm^2/s to less than 5×10^{-12} cm^2/s. Fluid-phase measurements (above T_m), however, gave highly reproducible results and consistently well-behaved redistributions of fluorescence. The temperature dependence of the diffusion coefficient from 25 to 35 °C is shown in Figure

[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; FITC, fluorescein isothiocyanate; T_m, phospholipid phase transition temperature; D, lateral diffusion coefficient.

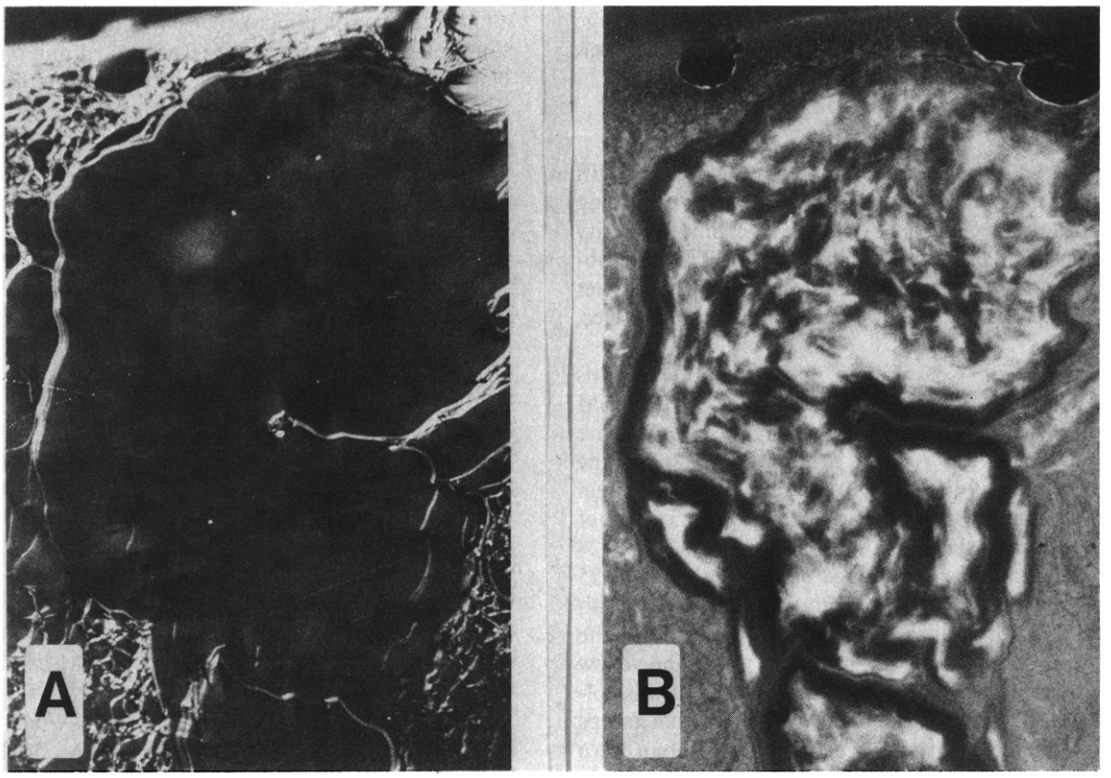

FIGURE 1: Photomicrographs of an oriented region of a DMPC multibilayer sample containing fluorescent-labeled M-13 coat protein (see text). Sample is in solid phase (below 23 °C). (A) Illumination with visible light by using Nomarski optics. (B) Fluorescence. The view is of the order of 500 × 800 μm. The photomicrographs illustrate the nonuniform distribution of the fluorescent-labeled M-13 coat protein (B) in seemingly well oriented multibilayers (A).

2, together with published data for lipid probes (Sheats & McConnell, 1978; Wu et al., 1977).

The addition of 0.5 mol % lysolecithin or 0.5 mol % sodium cholate to the samples did not affect the diffusion of M-13 coat protein above T_m. In addition, the diffusion was found to be independent of the concentration of coat protein in the range 500:1 to 2000:1 lipid to protein mole ratio. It is therefore unlikely that traces of cholate or lysolecithin or variations in the protein concentration could have had substantial effects upon the results.

Evidence for a specific association between M-13 coat protein and bacterial cardiolipin has been reported (Chamberlain & Webster, 1976). Evidence against such an association has been obtained in recent work with a mutant of *Escherichia coli* deficient in cardiolipin synthesis (Pluschke et al., 1978). We have determined the diffusion rate of coat protein in DMPC multibilayers containing 1 mol % cardiolipin to be the same as that in pure DMPC to within a factor of 2.

Due to the nonuniformity of the multibilayers below the phospholipid phase transition temperature, T_m, solid-phase measurements were carried out on giant liposomes. Above T_m, stripes of period 5.3 μm disappeared in less than 1 s, giving $D_{fluid} > 7 \times 10^{-9}$ cm^2/s, consistent with the multibilayer results. Upon cooling below T_m, we measured values of D, ranging from 10^{-10} cm^2/s to less than 5×10^{-12} cm^2/s, with the smaller values being obtained after longer periods at the low temperature (i.e., redistribution of fluorescence slows down as a function of time in the solid phase). The decay of stripe amplitude was often observed not to conform to a single exponential form, indicating a distribution of mobilities for the fluorescent protein molecules. Below T_m the liposomes sometimes show patches and swirls of fluorescence similar to those observed in multibilayers, whereas above T_m the fluorescence was always uniform. A comparison of observations under fluorescence and under visible illumination suggests that the change in distribution of fluorescence was a property of the M-13 coat protein rather than some artifact associated with the configuration of the multibilayers.

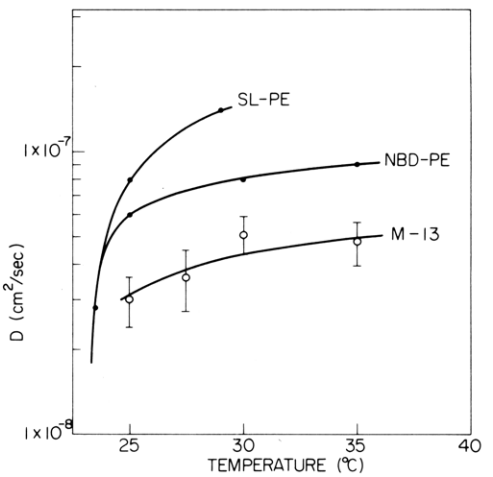

FIGURE 2: Lateral diffusion coefficient vs. temperature for the diffusion of molecules incorporated into DMPC multibilayers: spin-labeled phosphatidylethanolamine (SL-PE) (Sheats & McConnell, 1978); NBD-PE, fluorescent-labeled phosphatidylethanolamine (Wu et al., 1977); M-13, fluorescent-labeled M-13 phage coat protein. Data for NBD-PE estimated from plot in Wu et al. (1977). Recent measurements on NBD-PE in this laboratory have yielded diffusion coefficients of the order of 20% less than those given in this figure (Rubenstein et al., 1979). Error bars on M-13 data points represent standard deviations of an average of five measurements at each temperature.

Discussion

Our main conclusions from this work are that (1) above the phospholipid phase transition temperature, T_m, the coat protein diffuses almost as rapidly as the phospholipids and exhibits

a similar temperature dependence (Figure 2) and (2) the protein is nonuniformly distributed in the solid phospholipid phase (Figure 1), as a result of either protein aggregation or phase separation into protein-rich and protein-poor regions. This type of behavior has been reported previously (Kleemann & McConnell, 1976).

The measurement of the diffusion coefficient for well characterized proteins in model membranes will allow theories for two-dimensional diffusion to be tested. Saffman & Delbrück (1975) have proposed a hydrodynamic theory which predicts among other things a weak dependence of diffusion coefficient on particle radius and a reciprocal dependence on the thickness of the bilayer for a cylinder which spans the bilayer (Saffman & Delbrück, 1975). We suggest that there should be roughly a factor of 2 difference in the diffusion coefficient between otherwise similar molecules where one molecule spans only half the bilayer (e.g., a phospholipid molecule) and the other spans the entire bilayer (coat protein) and that this is the major source of the difference in the diffusion coefficients between M-13 coat protein and phospholipid.

The observations in the solid phase of both multibilayer samples and liposomes show a nonuniform distribution of the protein. Both the slowing of diffusion with time and the existence of both mobile and very slowly diffusing populations are consistent with the gradual formation of highly associated species.

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