Influence of the Membrane Glycoprotein and Cholesterol of Vesicular Stomatitis Virus on the Dynamics of Viral and Model Membranes: Fluorescence Studies†

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ABSTRACT: The interaction of the integral membrane glycoprotein of vesicular stomatitis virus with the phospholipid bilayer in reconstituted lipid vesicles was determined by differential polarized phase fluorometry, steady-state fluorescence anisotropy, and emission lifetime measurements. The glycoprotein was isolated from the viral membrane by solubilization with octyl β-glucopyranoside and was reconstituted into lipid vesicles by the detergent dialysis method. The fluorescent probes 1,6-diphenyl-1,3,5-hexatriene and 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene revealed that, in dipalmitoylphosphatidylcholine vesicles, the glycoprotein disorders the bilayer in the gel phase and orders it to a small extent in the liquid-crystalline phase. When the viral glycoprotein was reconstituted into 1-palmitoyl-2-oleoylphosphatidylcholine vesicles, only the ordering effect of the protein on the lipid bilayer was observed throughout the temperature range studied. The lifetime of both probes was found to be slightly shortened in the presence of the protein. No large effect of glycoprotein on the dynamics of the lipid phase was observed when the protein was reconstituted into total viral lipid vesicles. The role that cholesterol plays in determining the structural order of the viral membrane was determined by steady-state fluorescence anisotropy and differential polarized phase fluorometry. In intact viral membranes, as well as in viral lipid vesicles, the depolarization rotations of both 1,6-diphenyl-1,3,5-hexatriene and 1-[4-(trimethylammonio)-phenyl]-6-phenyl-1,3,5-hexatriene were highly hindered. Removal of cholesterol from the viral membrane, or from total viral lipid vesicles, disordered the membrane matrix markedly. Steady-state fluorescence anisotropy of *trans*-parinaric acid embedded in the viral envelope showed a broad phase separation in the viral membrane following depletion of cholesterol from the viral envelope. A similar effect was noted in cholesterol-free viral lipid vesicles. The results presented in this paper therefore demonstrate that both the membrane glycoprotein and cholesterol of vesicular stomatitis virus affect the dynamics of lipid bilayers to a significant extent. The effect of cholesterol was found to be greater than that of the protein. The role of cholesterol in determining the organization of glycoprotein spikes in the membrane of the virus and its implication for viral infectivity are discussed in light of these results.

Biological membranes of most eukaryotic cells and enveloped viruses are composed of phospholipids, glycolipids, sterols, and protein. In recent years, many efforts have been directed toward understanding the role of these components in determining the physical and biological properties of the intact membranes of rather complicated eukaryotic cells (Quinn & Chapman, 1980). In this paper, we have utilized the relatively simple, well-characterized biological membrane of vesicular stomatitis virus (VSV)[1] to study the interaction of lipids and protein in the bilayer. Vesicular stomatitis virions consist of a nucleocapsid core and a limiting membrane derived from the plasma membrane of infected host cells (Patzer et al., 1979; Wagner, 1975). The virion has five proteins, three of which are associated with the RNA to form an enzymatically active nucleocapsid core, whereas the membrane has only two proteins (Wagner, 1975). The externally oriented glycoprotein (G-protein) is an integral membrane protein which forms the spikes of the virus while the matrix (M) protein appears to line the inner surface of the viral membrane (Schloemer & Wagner, 1975; Zakowski & Wagner, 1980). The glycoprotein has a molecular weight of ~69 000 of which a 6000-dalton portion is a protease-resistant fragment embedded in the bilayer (Schloemer & Wagner, 1975). The sequence of a cDNA clone of the 3′ end of G-protein mRNA has provided clear evidence that the glycoprotein has 20 consecutive hydrophobic amino acids that traverse the viral membrane followed by 29 amino acids at the carboxy terminus that protrude from the inner surface (Rose et al., 1980; Rose & Gallione, 1981).

The lipid composition of the viral membrane is well-defined (Patzer et al., 1979). Cholesterol is an important component of the envelope, representing nearly 35 mol % of total viral lipids. Recent evidence from this laboratory has shown that cholesterol is distributed asymmetrically in the membrane bilayer and plays a significant role in determining viral infectivity (Moore et al., 1978; Patzer et al., 1978; Pal et al., 1981).

The purified glycoprotein of VSV has been reconstituted into phospholipid vesicles by the detergent dialysis method (Petri & Wagner, 1979). Recent studies in this laboratory have utilized the glycoprotein-reconstituted vesicles as a model system to study the interaction of the transmembrane protein with lipids in the bilayer (Petri et al., 1981); examination of the thermotropic behavior of the DPPC vesicles reconstituted with the glycoprotein of VSV has revealed a profound effect of the G-protein on lipid phase behavior (Petri et al., 1980). Furthermore, steady-state fluorescence anisotropy studies using four different fluorophores, presumably residing in different

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[1] Abbreviations: VSV, vesicular stomatitis virus; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; G-protein, glycoprotein; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene; SPM, sphingomyelin; MLV, multilamellar vesicle(s); TPA, *trans*-parinaric acid; Tris, tris(hydroxymethyl)aminomethane.

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regions of the bilayer, have also demonstrated lowering of the phase transition temperature of the lipid matrix in G-protein-reconstituted vesicles (Petri et al., 1981).

In recent years, fluorescence polarization techniques have provided much useful information about the structure and dynamics of membrane lipids. Steady-state fluorescence anisotropy has been interpreted exclusively in terms of microviscosity of the membrane (Shinitzky & Barenholz, 1978). More recently, complex patterns of motion of the fluorescent probes in lipid bilayers have been obtained by using time-resolved fluorescence anisotropy and differential polarized phase fluorometric measurements (Kinosita et al., 1977; Lakowicz et al., 1979). These studies have demonstrated that the degree of rotation of fluorophores in membranes is restricted by the molecular packing of lipids in the bilayer and this in turn can be correlated with the structural order of the lipids in the membrane. In this paper, a combination of differential polarized phase fluorometry, steady-state fluorescence anisotropy, and emission lifetime measurements of various membrane-bound fluorophores have been used to study the effect of cholesterol and glycoprotein on the dynamic behavior of lipids in viral and model membranes. Our results have demonstrated that the structural order parameter calculated from differential polarized phase fluorometric measurements is strongly influenced by the presence of both glycoprotein and cholesterol in the bilayer.

Experimental Procedures

Materials

Lipids and Fluorescent Probes. 1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) were obtained from Avanti Biochemical, Birmingham, AL. Each lipid stock yielded a single spot on silica gel thin-layer chromatography plates (E. Merck, Darmstadt, Germany) run in a chloroform/methanol/ammonia (65:25:4) solvent system. 1,6-Diphenyl-1,3,5-hexatriene (DPH), 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH), and *trans*-parinaric acid (TPA) were obtained from Molecular Probes, Junction City, OR. The probes were dissolved in tetrahydrofuran (10 mM) and were stored at −70 °C in the dark until used.

Cells and Virus. Baby hamster kidney (BHK-21) cells were grown at 37 °C in Dulbecco's modified Eagle's (DME) medium supplemented with 10% tryptose/phosphate broth, 10% calf serum, and antibiotics as described elsewhere (Barenholz et al., 1976). Plaque-purified vesicular stomatitis virus of the Indiana serotype was used to infect the cell monolayer at a multiplicity of 0.1 plaque-forming unit/cell. Bullet-shaped virions were harvested at 21-h postinfection and were purified by differential, rate zonal, and equilibrium centrifugation (Barenholz et al., 1976). Purified virions were stored in phosphate-buffered saline (pH 7.4) at a concentration of 2–5 mg/mL at −70 °C until further use.

Methods

Isolation of the VSV G-Protein and Reconstitution into Lipid Vesicles. The glycoprotein was isolated from VS virions by solubilization with octyl glucopyranoside as described by Petri & Wagner (1979). After purification through a 15–30% sucrose gradient containing 60 mM octyl glucoside, the isolated glycoprotein was reconstituted into DPPC vesicles or POPC vesicles as described elsewhere (Petri et al., 1980, 1981). The glycoprotein was also reconstituted into vesicles made of extracted endogenous viral lipids. For this purpose, VS virion suspensions (1 mg/mL) were dialyzed against Tris-HCl buffer (pH 7.5) to remove salt and were then treated with octyl glucoside (30 mM) for 1 h at room temperature to release the glycoprotein from the lipid envelope. The solution was then centrifuged onto a glycerol pad for 90 min at 100000*g* in the SW50.1 rotor to remove nucleocapsid and any contaminating M protein. The supernatant was carefully collected and dialyzed against 50 mM KCl for 36 h. The solubilized G-protein was reconstituted spontaneously into endogenous viral lipids following dialysis. For production of viral lipid vesicles without glycoprotein, the lipids from the viral suspension (1 mg/mL) were extracted from chloroform/methanol (2:1 v/v) (Folch et al., 1957), and octyl glucoside (30 mM) was added to the chloroform extract; the solvent was then evaporated, lyophilized, and resuspended in 10 mM Tris buffer, pH 7.5, and dialyzed for 36 h against 50 mM KCl. We could extract nearly 95% of the viral lipids either by detergent or by organic solvents.

Lipid and Protein Analysis. The mole percent of glycoprotein in the vesicles was calculated by assuming a molecular weight of 69 000 for G-protein. The phospholipid content was estimated by the method of Marinetti (1962), and the protein concentration was assayed as described by Lowry et al. (1951). Cholesterol concentrations were determined by using cholesterol oxidase as described elsewhere (Patzer et al., 1978).

Depletion of Cholesterol from Viral Membrane. Cholesterol was depleted from viral membrane by incubating virions for 16 h with lipoproteins in serum enriched with sphingomyelin (SPM) for 16 h, as described by Pal et al. (1981). In short, 1 volume of bovine brain sphingomyelin (2–3 mg/mL) in a 5:1 mixture of tetrahydrofuran and 0.6% KCl (v/v) was added to 10 volumes of vigorously stirred Eagle's basal medium containing 10% calf serum. The solution after freezing was lyophilized, and the cholesterol-depleting medium was prepared by dissolving the lyophilized medium in appropriate volumes of sterile distilled water. Purified VSV (200 μg) was incubated with 2 mL of sphingomyelin-enriched lipoprotein medium for 16 h, and the virions were repurified by sucrose-gradient centrifugation as described elsewhere (Pal et al., 1981). Such treatment of virions with SPM-enriched serum depleted nearly 75% of the cholesterol from the membrane of the virus. Lipids were extracted from the virions by the method of Folch et al. (1957). Cholesterol was removed from total lipid extracts by thin-layer chromatography with hexane/ether/acetic acid (30:6:0.5 v/v/v) as the solvent system. Phospholipids free of cholesterol were eluted from the thin-layer plate with methanol/chloroform (95:5 v/v).

Fluorescence Studies. So that more detailed information on bilayer structure and dynamics could be obtained, the following three fluorescent techniques were used in this study.

(*A*) *Steady-State Fluorescence Depolarization.* Steady-state fluorescence depolarization experiments were performed exactly as described by Barenholz et al. (1976), by using a modified Perkin-Elmer MPF3 spectrofluorometer with polarizers in the excitation and emission beams. Cuvette temperature was monitored continuously by means of a Yellow Springs Instrument thermistor probe connected to a digital ohmmeter. When fluorescence depolarization was measured as a function of temperature, the sample was heated to 60 °C and then cooled at a rate of 25 °C/h. The fluorescence intensities parallel and perpendicular to the excitation beam, as well as the temperature, were measured during cooling.

Three different fluorescent probes were used. The use of DPH as a fluorescent probe specific for the hydrophobic region of the lipid bilayer has been described elsewhere (Shinitzky & Barenholz, 1978; Lakowicz, 1981). The use of TMA-DPH as a fluorescent membrane probe has been described by

Table I: Lifetimes (τ) and Differential Tangents (tan Δ) of DPH and TMA-DPH in Lipid Vesicles with or without G-Protein

temp (°C)	DPH				TMA-DPH			
	lipid alone		lipid + G-protein		lipid alone		lipid + G-protein	
	τ (ns)	tan Δ	τ (ns)	tan Δ	τ (ns)	tan Δ	τ (ns)	tan Δ
				(A) DPPC Vesicles				
50	7.50	0.110	6.75	0.095	3.05	0.069	2.60	0.05
45	7.85	0.120	7.20	0.095	3.50	0.075	3.05	0.06
40	8.50	0.110	7.25	0.087	4.50	0.057	3.60	0.06
35	9.10	0.040	8.10	0.065	5.45	0.025	4.30	0.042
30	10.15	0.030	8.75	0.050	5.70	0.015	4.60	0.038
25	10.25	0.027	9.10	0.045	5.80	0.010	4.75	0.025
				(B) POPC Vesicles				
60	6.21	0.135	5.51	0.130	2.55	0.070	2.56	0.064
45	6.37	0.150	5.75	0.141	2.85	0.075	2.67	0.069
35	6.84	0.161	5.99	0.150	3.19	0.085	2.96	0.075
30	7.04	0.175	6.18	0.161	3.60	0.090	3.30	0.080
25	7.19	0.185	6.37	0.170	*a*	*a*	*a*	*a*
20	7.37	0.190	6.37	0.171	4.24	0.100	4.02	0.081
15	7.56	0.188	6.70	0.160	4.77	0.097	4.42	0.066

[a] Not determined.

Prendergast et al. (1981). This probe is similar to DPH in fluorescence properties but occupies a more defined region in the bilayer due to the cationic trimethylammonium moiety which is attached to the para position of one of the phenyl rings. *trans*-Parinaric acid is a naturally occurring fluorescent fatty acid which preferentially partitions into, and reports on, gel-state phospholipids (Sklar et al., 1975).

Fluorescence measurements of DPH and TMA-DPH were performed by excitation at 360 nm; fluorescence was detected by monitoring the emission at 430 nm, by using the MPF3 390-nm cutoff filter to reduce scattered light. The fluorescence of vesicle preparations containing TPA was measured in a similar fashion, except that TPA was excited at 320 nm and the emission monitored at 420 nm by using the MPF3 410-nm cutoff filter to reduce scattered light.

(*B*) *Emission Lifetime.* Emission lifetime was measured directly in an updated version of the cross-correlation phase and modulation fluorometer from SLM Instruments, Urbana, IL. For DPH and TMA-DPH, the excitation wavelength (360 nm) was selected from a 450-W xenon arc lamp by a monochromator (0.5-mm band path) and modulated at 30 MHZ by a Sears-Debye ultrasonic light modulator. Lifetime was measured both by phase shift and by the demodulation of the fluorescence of the probe-loaded vesicle suspension relative to a scattering suspension of DPPC-fused unilamellar vesicles of approximately equal emission intensity (Spencer & Weber, 1969). Measurements were made continuously until stable values were obtained.

(*C*) *Differential Phase Fluorescence Measurement.* The theory and experimental techniques of differential phase fluorometry have been described elsewhere (Lakowicz & Prendergast, 1978; Lakowicz et al., 1979). The differential lifetime of vertically and horizontally polarized components of the modulated fluorescence was measured on the same instrument as used for emission lifetime measurement. The nonzero limiting anisotropy (r_∞) at times long compared to the fluorescence lifetime was calculated by the combined use of lifetime, differential lifetime, and steady-state fluorescence anisotropy measurements as described by Lakowicz et al. (1979) and Lakowicz (1981).

Results

Comparative Dynamics of Lipids in Detergent-Dialyzed Vesicles Reconstituted with or without VSV Glycoprotein. Using DPH and TMA-DPH as fluorescent probes, we set out to determine the structural order of lipids in G-protein-reconstituted vesicles. The effect of G-protein on the gel and liquid-crystalline states of the lipid bilayers was determined by reconstitution of the glycoprotein into three different types of lipid vesicles as well as into reconstituted total viral lipids, which contain nearly 35 mol % cholesterol. This latter experiment with vesicles made of viral lipids was performed in order to examine whether the G-protein can influence the dynamics of the lipid bilayer in the presence of cholesterol. Emission lifetime of DPH and TMA-DPH and their differential phase behavior in lipid bilayers with or without glycoprotein were determined to calculate the limiting anisotropy as described by Lakowicz et al. (1979), as recounted below.

(*A*) *Emission Lifetime.* Table I compares the average lifetime values of both DPH and TMA-DPH in vesicles reconstituted by detergent dialysis of DPPC or POPC with or without G-protein. The presence of glycoprotein in the vesicles reduces the average lifetime for both probes in the bilayer. Such shortening of lifetime in the presence of G-protein may be due to a change in the homogeneity of the system which results in the appearance of a shorter lifetime fluorophore species; however, an increase in the nonradiative process, such as interaction with water, may also decrease the lifetime of the probes in the presence of the protein (Lakowicz, 1981). The temperature affects the lifetime of the probes in the vesicles with or without G-protein. However, the effect was more pronounced in DPPC vesicles as the lipids are undergoing phase transition in the temperature range studied here. The lifetime of DPH in total viral lipid vesicles with or without G-protein was not significantly affected either by temperature or by the presence of G-protein, and the average lifetime values varied from 9.2 to 10.3 ns between 40 and 20 °C, respectively (data not shown). The presence of nearly 35 mol % cholesterol in these vesicles may explain this result.

(*B*) *Differential Polarized Phase Fluorometry.* Table I also compares as a function of temperature the differential tangent (tan Δ) of DPH and TMA-DPH in DPPC or POPC vesicles reconstituted with or without the VSV glycoprotein. The presence of G-protein in the vesicles significantly broadened the profile during the transition and resulted in a somewhat lower value of tan Δ in the liquid-crystalline state and a higher value in the gel phase compared to lipid vesicles alone. The tan Δ values of DPH and TMA-DPH in POPC vesicles are

Table II: Lifetime (τ), Differential Tangents (tan Δ), and Theoretically Calculated Differential Tangents (calcd tan Δ_{max}) of DPH and TMA-DPH in Intact VSV, Cholesterol-Depleted VSV, Multilamellar Vesicles Made of Total Viral Lipids, and Multilamellar Vesicles (MLV) of Cholesterol-Free Viral Lipids[a]

temp (°C)	intact VSV			cholesterol-depleted VSV		MLV total viral lipids			cholesterol-free MLV viral lipids		
	τ (ns)	tan Δ	calcd tan Δ_{max}	τ (ns)	tan Δ	τ (ns)	tan Δ	calcd tan Δ_{max}	τ (ns)	tan Δ	calcd tan Δ_{max}
					(A) DPH						
46	8.60	0.080	0.297	8.39	0.093	7.12	0.053	0.257	6.50	0.118	0.232
36	8.97	0.062	0.304	8.91	0.080	7.20	0.056	0.267	6.92	0.135	0.263
31	9.15	0.063	0.309	9.09	0.079	7.30	0.056	0.272	7.04	0.142	0.261
					(B) TMA-DPH						
45	5.10	0.035	0.213	*b*	*b*	4.10	0.035	0.172	2.39	0.050	0.113
40	5.39	0.029	0.222	*b*	*b*	4.83	0.035	0.205	2.91	0.065	0.135
35	5.62	0.028	0.229	*b*	*b*	5.05	0.036	0.210	3.29	0.072	0.150

[a] The tan Δ_{max} was calculated as described elsewhere (Lakowicz et al., 1979). [b] Not determined.

also shown in Table I. In the temperature range studied, the lipids remain in the liquid-crystalline phase. The tan Δ values tend to reach a maximum point around 20 °C and slowly drop below that temperature. Insertion of the glycoprotein in the vesicles tends to lower the tan Δ value at all temperatures studied without affecting the profile significantly. The tan Δ values of DPH in total viral lipid vesicles with or without G-protein were nearly identical at all temperature ranges studied (data not shown).

Figure 1 compares the limiting anisotropy (r_∞) of DPH (Figure 1A) and TMA-DPH (Figure 1B) in vesicles composed of DPPC, POPC, or total viral lipids reconstituted with or without VSV G-protein. It is clear from Figure 1 that in DPPC vesicles the glycoprotein increased the r_∞ and hence the structural order of lipids in the liquid-crystalline state, whereas in the gel state it tended to decrease the r_∞ value by disordering the bilayer significantly. In POPC vesicles, where the lipids are in the liquid-crystalline state throughout the temperature range studied, only the ordering effect of the protein on the bilayer was observed at all temperatures. The limiting anisotropy (r_∞) of DPH in total viral lipid vesicles with or without the glycoprotein is also shown in Figure 1A. Interestingly, the G-protein had only a slight effect on the lipid order parameter in these vesicles.

These results demonstrate that the glycoprotein of VSV affects the dynamic properties of the lipid bilayer in reconstituted vesicles to a significant extent. The order of the lipid matrix increased in the presence of G-protein in the liquid-crystalline state, whereas the protein disordered the lipid bilayer in the gel state. No specific effect of G-protein on membrane dynamics was observed in vesicles prepared from total viral lipids, which contain nearly 35 mol % cholesterol.

Effects of Cholesterol on Dynamics of Viral and Model Membranes. In this paper, we have studied the hindered rotation of DPH and TMA-DPH in the VSV membrane by differential polarized phase fluorometry in order to determine whether cholesterol in the membrane could affect the structural order of the bilayer. Further, the possibility of any lateral phase separation in the membrane following depletion of cholesterol was also examined by the use of the fluorescent probe *trans*-parinaric acid.

(A) Emission Lifetime. Table II compares the average lifetime of DPH and TMA-DPH in intact VSV, cholesterol-depleted VSV, MLV made of total viral lipids, and MLV made of cholesterol-free viral lipids. The average lifetime of TMA-DPH is seen to be shorter than that of DPH in the viral membrane and in lipid vesicles. The lifetime of both probes decreased in the following order: intact VSV > cholesterol-depleted VSV > total lipid vesicles > cholesterol-free viral lipid

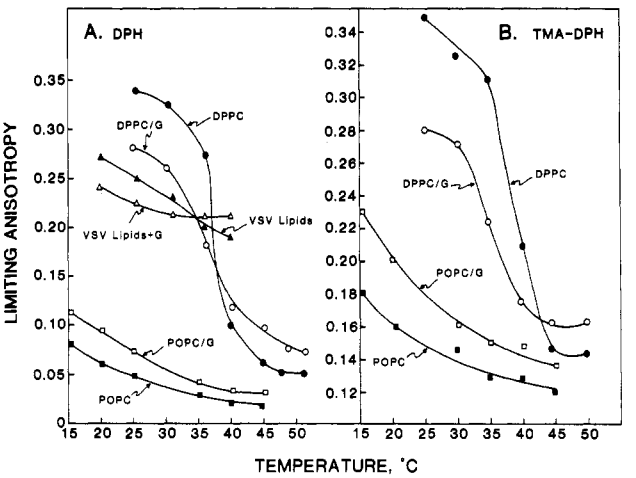

FIGURE 1: Limiting anisotropy as a function of temperature for (A) DPH and (B) TMA-DPH in DPPC, POPC, or total VSV lipid vesicles reconstituted with or without the glycoprotein of VSV. Limiting anisotropies at different temperatures were calculated from differential tangents, lifetime, and steady-state anisotropy as outlined under Experimental Procedures and described elsewhere (Lakowicz et al., 1979). The detergent-dialyzed vesicles consisted of POPC containing either no glycoprotein (■) or 0.5 mol % glycoprotein (□), DPPC containing either no glycoprotein (●) or 0.5 mol % glycoprotein (○), and total viral lipid vesicles containing either no glycoprotein (▲) or 0.85 mol % glycoprotein (△).

vesicles. It is to be noted that the depletion of cholesterol (by ~75%) from the viral membrane had little effect on the lifetime of DPH embedded in the bilayer. However, in cholesterol-free viral lipid vesicles, lifetimes of both probes were much shorter. The higher lifetime of DPH in viral membrane may be due to the heterogeneity of the system created by the presence of cholesterol and protein in the bilayer.

(B) Differential Polarized Phase Fluorometry. The differential tangents (tan Δ) of DPH and TMA-DPH in intact VSV, cholesterol-depleted VSV, total viral lipid MLV, and cholesterol-free viral lipid MLV are also shown in Table II. The observed value of tan Δ in all these samples was nearly 30–50% of the theoretically calculated tan Δ_{max}. This suggests that in viral membrane and in viral lipid vesicles the depolarization rotations of DPH and TMA-DPH are highly hindered as was noted by Lakowicz et al. (1979). It should also be noted that the removal of cholesterol from the viral membrane or from total viral lipid vesicles increased the tan Δ value, which is a reflection of decreased hindrance to the motion of the probe in the bilayer. The effect of depletion of cholesterol on tan Δ was greater in cholesterol-free lipid vesicles than in the intact viral membrane. The presence of G-protein in the

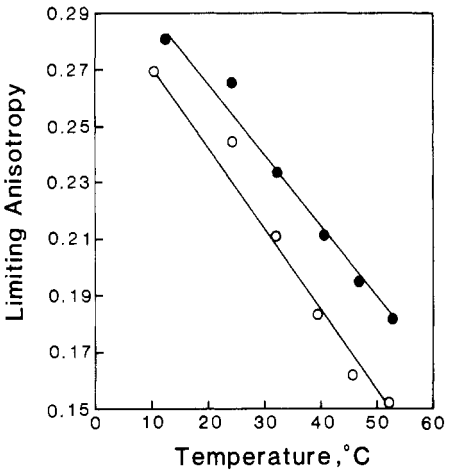

FIGURE 2: Limiting anisotropy as a function of temperature for DPH in control VSV (●) and in cholesterol-depleted VSV (○). Cholesterol was depleted from the membrane of the virions with sphingomyelin-enriched serum lipoprotein as described under Experimental Procedures. Such treatment resulted in nearly 75% depletion of cholesterol from the virion membrane. DPH was added to the virus suspension in phosphate-buffered saline (pH 7.4) at a probe to lipid ratio of 1:180. Limiting anisotropy at different temperatures was calculated from differential tangents, lifetime, and steady-state anisotropy measurements as described by Lakowicz et al. (1979).

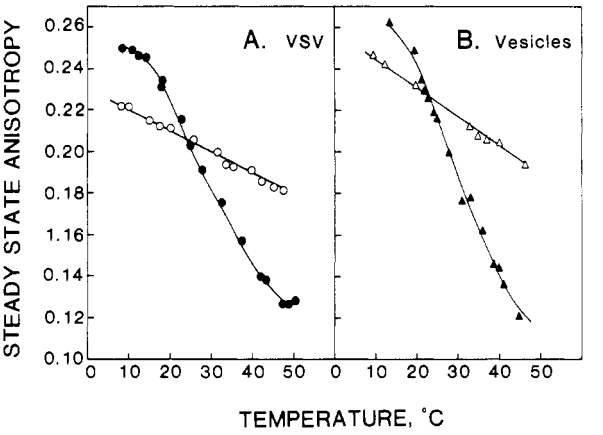

FIGURE 3: Steady-state fluorescence anisotropy of *trans*-parinaric acid as a function of temperature in (A) control VSV (○) and cholesterol-depleted VSV (●) and in (B) total viral lipid multilamellar vesicles (△) and cholesterol-free viral lipid MLV (▲). Nearly 75% of cholesterol was depleted from the membrane of VSV by incubating with sphingomyelin-enriched serum lipoprotein as described under Experimental Procedures. Cholesterol was completely removed from the total viral lipid extract as described under Experimental Procedures. *trans*-Parinaric acid was added to the virion suspension and multilamellar vesicles at a probe to lipid ratio of 1:200 and sealed under nitrogen. The steady-state fluorescence anisotropy at different temperatures was measured as described by Barenholz et al. (1976).

envelope of VSV may explain such a difference in the effect of cholesterol depletion on tan Δ in the VSV envelope and in lipid vesicles.

Figure 2 compares the limiting anisotropy of DPH in intact VSV and the viral membrane depleted of 75% cholesterol calculated from observed values of lifetime, steady-state anisotropy, and differential tangent. It is to be noted that the limiting anisotropy made a significant contribution to the steady-state anisotropy in the viral membrane. However, the depletion of cholesterol decreased the r_∞ value throughout the temperature range studied. This suggests that cholesterol affects the order of the lipid bilayer in the viral membrane and removal of cholesterol tends to decrease the structural order of the lipid matrix significantly.

(*C*) *Steady-State Anisotropy Studied with trans-Parinaric Acid.* Figure 3 compares the temperature-dependent steady-state fluorescence anisotropy of *trans*-parinaric acid in normal and cholesterol-depleted virions (Figure 3A) and in MLV of total viral lipids with or without cholesterol (Figure 3B). A broad gel–liquid phase transition was observed in the cholesterol-depleted virion envelope. This is also evident from the fact that at temperatures above 25 °C, the steady-state anisotropy of *trans*-parinaric acid was lower in cholesterol-depleted virions compared to that of control virions and was higher at temperatures below 25 °C. This suggests that cholesterol tends to rigidify the VSV membrane above 25 °C while it has a fluidizing effect below 25 °C. Similar effects of cholesterol on membrane fluidity were observed in lipids undergoing phase transition (DeKruijff et al., 1972). The steady-state anisotropies of *trans*-parinaric acid in viral lipid MLV with or without cholesterol are also presented in Figure 3B. As was the case in intact viral membranes, removal of cholesterol from the viral lipid mixture also led to a broad phase transition in the membrane.

Discussion

The glycoprotein spikes of the VSV envelope are responsible for the initial attachment of the virus to the cell plasma membrane during the initial stages of viral infection (Bishop et al., 1975). The G-protein is an intrinsic membrane protein which spans the lipid bilayer by a sequence of 20 hydrophobic amino acids (Rose et al., 1980). Reconstituted lipid vesicles containing the glycoprotein serve as a useful model to study lipid–protein interaction in biological membranes (Petri & Wagner, 1979). It is clear from the limiting anisotropy values of DPH and TMA-DPH presented here that the G-protein in DPPC vesicles increases the order of the membrane in the liquid-crystalline state and disorders it in the gel state. In POPC vesicles, insertion of the G-protein in the bilayer increases the order of the membrane matrix to a significant extent. Indeed, a similar ordering effect by G-protein was also observed in egg phosphatidylcholine vesicles as determined by electron spin resonance spectroscopy (Altstiel & Landsberger, 1981). Incorporation of G-protein into total viral lipid vesicles containing nearly 35 mol % cholesterol resulted in little effect on the order of the lipid bilayer.

The ordering effect of the VSV G-protein in the fluid state of lipids and the disordering effect on the gel state have also been observed for integral proteins in other systems (Marsh et al., 1978; Gomez-Fernandez et al., 1979; Susi et al., 1979). Studies by deuterium nuclear magnetic resonance, on the other hand, indicated that proteins generally decrease the order of the lipid bilayer in the fluid state of the membrane (Seelig et al., 1981). A number of theoretical models have been put forward to provide a consistent interpretation of these results. In one such model (Chapman, 1982; Pink et al., 1981), it was proposed that part of the lipids in the bilayer can be trapped between two or three protein molecules. At temperatures greater than the main transition temperature, the phospholipid molecules that are trapped within the clusters of intrinsic protein have the ends of their hydrocarbon chains more statically disordered than those of the lipids far from the protein. It was also suggested that below the transition temperature the lipid chain crystallizes and proteins are squeezed out, which results in the formation of eutectic mixtures of protein and lipids (Chapman et al., 1979). Fluorescent probe molecules may tend to concentrate in these protein–lipid patches, and when the temperature is raised, a marked increase of mobility below the lipid transition temperature would occur mainly at the eutectic melting temperature. In a more recent study, Jahnig et al. (1982) have suggested that the protein

induces a tilt of the preferred axes of lipid orientation and increases the lipid orientational order with respect to these tilted axes. In a mellitin protein reconstituted system using DPH as a fluorophore, they observed a considerable increase in the rigid-body orientational order of the lipid chains in the fluid state with relatively no effect on the ordered state. It was concluded that the total orientational order of the lipid bilayer should be divided into fast and slow components. The fast order involves the local fluctuations of the lipid chain orientation which could be measured in the time scale of fluorescence anisotropy. This order was found to be increased by protein. The slow order, on the other hand, involves the fluctuation of the preferred axes of orientation due to the lateral diffusion of lipids and is decreased by protein.

The structural and biological function of sterol in membranes has been extensively investigated (Cooper, 1977; Huang, 1977; Estep, et al., 1978; DeKruijff et al., 1972). Due to the significant role played by cholesterol in the envelope of vesicular stomatitis virus (Pal et al., 1980, 1981), we investigated how the presence of cholesterol in the viral membrane determines the order of the bilayer. Differential polarized phase fluorometric studies have shown here that the depolarizing rotations of both DPH and TMA-DPH are highly hindered in the viral membrane and the structural parameter related to the limiting anisotropy (r_∞) is a major contributor to the steady-state anisotropy. A moderately decreased order of the viral membrane was observed following partial depletion (~75%) of cholesterol from viral membrane. This effect was more magnified in viral lipid vesicles. TMA-DPH and DPH tend to partition into both gel and liquid-crystalline lipid phases equally well and thus could not detect the presence of any gel-state lipids in the viral membrane following depletion of cholesterol. *trans*-Parinaric acid has been shown to partition preferentially in the gel-state phospholipid (Sklar et al., 1975; Rintoul et al., 1979). Using this probe, we have detected a broad phase transition following depletion of cholesterol from viral membrane or from total viral lipid vesicles. The formation of such a gel state in the VSV membrane has been attributed to the presence of nearly 25 mol % sphingomyelin in the envelope which has a transition temperature of 37 °C. *trans*-Parinaric acid, due to its preferential partitioning in the sphingomyelin gel state, could detect the transition of this lipid which DPH or TMA-DPH could not detect. Indeed, a similar transition of sphingomyelin could be detected in POPC vesicles by *trans*-parinaric acid even when it is composed of only 10 mol % total lipids (data not shown).

The incorporation of VSV glycoprotein in total viral lipid vesicles containing cholesterol has a much smaller effect on the structural order of the lipid bilayer than in vesicles composed of phosphatidylcholine alone. It is quite conceivable that the organization of G-protein in the lipid bilayer may be affected by the presence of cholesterol in the membrane. Such an effect of cholesterol on VSV glycoprotein spikes may represent the key role of cholesterol in promoting viral infectivity rather than the ordering effect of cholesterol per se. This is supported by our earlier observation that reducing the degree of order of viral membrane lipids by treatment with phospholipase C has only a minor effect on viral infectivity as long as the concentration of membrane cholesterol remains unaltered (Moore et al., 1977). It is possible that the presence of cholesterol in the viral envelope determines the state of aggregation of G-protein in the membrane or controls its vertical displacement. Indeed, similar effects of cholesterol in determining the lateral organization of proteins in the plane of the membrane have been observed by Cherry et al. (1980). A possible role of cholesterol in regulating associations between integral membrane proteins has been studied recently in the membrane of human erythrocytes (Muhlebach & Cherry, 1982). It has been shown that the aggregation pattern of band 3 protein in erythrocyte membrane was affected by altering the cholesterol/phospholipid mole ratio in the membrane. Also, recent studies have demonstrated that the modulation of the membrane order parameter by cholesterol has a marked effect on the vertical displacement of membrane protein (Borochov et al., 1979).

Registry No. DPPC, 63-89-8; POPC, 26853-31-6; cholesterol, 57-88-5.

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Calmodulin-Dependent Protein Phosphatase: A Developmental Study†

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ABSTRACT: Calmodulin-dependent protein phosphatase, one of the major calmodulin-binding proteins in bovine brain, dephosphorylates casein with a specific activity of 15 nmol mg^{-1} min^{-1} at 30 °C. The stimulation of phosphatase activity by calmodulin is reversed by ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid or trifluoperazine, a calmodulin antagonist. Antibodies raised in rabbit against the phosphatase inhibit the enzyme activity. The levels of the protein in brain extracts from various animals, determined by a radioimmunoassay, range from 20 μg/g of tissue in chick and fish brains to 143 μg in rat cerebrum. The ontogeny of the phosphatase was studied in nervous tissues from rat and chick, animals in which synaptogenesis takes place at different times during their development. The levels of the protein increased significantly in rat cerebrum and cerebellum and in chick brain and retina during the periods corresponding to major synapse formation. In rat cerebrum, the enzyme appeared to be equally distributed between the cytosol and the particulate fraction; the level in both compartments increased during the major period of synapse formation. Thus, the development of calmodulin-dependent protein phosphatase closely parallels synaptogenesis, implicating a role in some synaptic function.

Calmodulin, a multifunctional Ca^{2+}-binding modulator protein, is a major intracellular receptor of Ca^{2+}, regulating a wide spectrum of enzymatic activities [for review, see Wang & Waisman (1979), Cheung (1980), Klee et al. (1980), Brostrom & Wolff (1981), and Means et al. (1982)]. Calmodulin-dependent protein phosphatase is a major calmodulin-binding protein in brain extracts, and it dephosphorylates various proteins, including inhibitor 1, phosphorylase *b* kinase, casein, and histone (Stewart et al., 1982; Yang et al., 1982). This enzyme, previously referred to as calcineurin or CaM-BP_{80}, is particularly rich in the neostriatum (Wallace et al., 1980a) where it is localized principally at postsynaptic sites within neuronal somata and dendrites (Wood et al., 1980a). In chick retina, it has been found in both pre- and postsynaptic terminals (Cooper et al., 1982). The protein is a heterodimer, with the large subunit (M_r 60 000) binding calmodulin in a Ca^{2+}-dependent manner (Richman & Klee, 1978; Sharma et al., 1979; Wang et al., 1980) and the small subunit (M_r 16 500) binding four Ca^{2+} with high affinity ($K_d \leq 10^{-6}$ M) (Klee et al., 1979).

As part of our long-range goal to define the multifunctional roles of calmodulin in biological systems, we studied the ontogeny of calmodulin-dependent protein phosphatase in developing nervous tissues from rat and chick. The results of this investigation demonstrate that the phosphatase levels increase during the time of major synapse formation. A preliminary report of these results has appeared (Tallant & Cheung, 1982).

Materials and Methods

Materials. ^{125}I (16–20 mCi/μg) was purchased from Amersham and [γ-^{32}P]ATP (2 mCi/mmol) from New England Nuclear. Sodium dodecyl sulfate ($NaDodSO_4$)[1] and Affi-Gel Blue were obtained from Bio-Rad, and iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) was from Pierce Chemical Co. Sigma Chemical Co. supplied Triton X-100, phenylmethanesulfonyl fluoride, leupeptin, aprotinin, bovine serum

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; CaM, calmodulin; Tris, tris(hydroxymethyl)aminomethane.

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