Characterization of the Fluorophore 4-Heptadecyl-7-hydroxycoumarin: A Probe for the Head-Group Region of Lipid Bilayers and Biological Membranes†

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ABSTRACT: The fluorophore 4-heptadecyl-7-hydroxycoumarin was used as a probe to study the properties of phospholipid bilayers at the lipid–water interface. To this end, the steady-state fluorescence anisotropy, the differential polarized phase fluorometry, and the emission lifetime of the fluorophore were measured in isotropic viscous medium, in lipid vesicles, and in the membrane of vesicular stomatitis virus. In the isotropic medium (glycerol), the probe showed an increase in the steady-state fluorescence anisotropy with a decrease in temperature, but the emission lifetime was unaffected by the change in temperature. In glycerol, the observed and predicted values for maximum differential tangents of the probe were identical, indicating that in isotropic medium 4-heptadecyl-7-hydroxycoumarin is a free rotator. Nuclear magnetic resonance and differential scanning calorimetric studies with lipid vesicles containing 1–2 mol % of the fluorophore indicated that the packaging density of the choline head groups was affected in the presence of the probe with almost no effect on the fatty acyl chains. The fluorophore partitioned equally well in the gel and liquid-crystalline phase of the lipids in the membrane, and the phase transition of the bilayer lipids was reflected in the steady-state fluorescence anisotropy of the probe. The presence of cholesterol in the lipid vesicles had a relatively small effect on the dynamics of lipids in the liquid-crystalline state, but a significant disordering effect was noted in the gel state. One of the most favorable properties of the probe is that its emission lifetime was unaffected by the physical state of the lipids or by the temperature. In the membrane of vesicular stomatitis virus, the fluorophore showed a large change in steady-state fluorescence anisotropy with temperature, but its lifetime remained unaffected. In lipid vesicles as well as in the virion membrane, the tangent defects were too large to be explained only by the anisotropy in the depolarizing rotations; hindered isotropic torsional motions of the probe also accounted for tangent defects. Depletion of cholesterol from the membrane of the virus decreased the steady-state fluorescence anisotropy of the hydroxycoumarin fluorophore embedded in the bilayer while no significant effect was noted in cholesterol-enriched virions. Limiting anisotropy (r_∞) of 4-heptadecyl-7-hydroxycoumarin was measured in liposomes reconstituted with either the integral glycoprotein or the peripheral matrix protein of vesicular stomatitis virus. The glycoprotein had little effect on the order of the lipid in the liquid-crystalline state but disordered the bilayer significantly in the gel state. The matrix protein, on the other hand, ordered the bilayer in the gel state. Incorporation of either of these proteins in the bilayer had no effect on the emission lifetime of the probe.

Fluorescence spectroscopic techniques have provided much useful information about the organization and dynamics of lipids in membranes (Yguerabide & Foster, 1981; Lakowicz, 1981). Extrinsic fluorescent probes are often used as reporters of the structural changes occurring in natural and model membranes. Measurements of fluorescence anisotropy are related to the rotational motion of the probe, reflecting the frictional retardation forces imposed by its microenvironment (Weber, 1953). These measurements have been widely used for the estimation of the "microviscosity" of the membranes (Shinitzky & Barenholz, 1978). In recent years, more complex patterns of the motion of fluorophore in lipid membranes 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 the probes in the membrane is restricted by the molecular packaging of lipids in the bilayer and this in turn can be correlated with the structural order of lipids in the membrane.

A number of fluorescent probes have been used in recent years to study the dynamics of lipids in the bilayer. 1,6-Diphenyl-1,3,5-hexatriene (DPH)[1] is extensively used as a probe to determine the structural parameter of membranes due to its favorable photophysical properties (Shinitzky & Barenholz, 1978; Lakowicz et al., 1981). A cationic analogue of DPH called 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) has been used recently as a probe for the hydrocarbon region near the lipid–water interface in the bilayer (Prendergast et al., 1981). Due to their rodlike shape and structural similarity to the membrane lipids, *n*-(9-anthroyloxy) fatty acids are likely to fit in the membrane with their acyl chains parallel to those of the phospholipids. These fatty acids have been used to estimate membrane fluidity gradients in the

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[1] Abbreviations: HC, 4-heptadecyl-7-hydroxycoumarin; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylammonio)-phenyl]-6-phenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; SPM, sphingomyelin; SUV, small unilamellar vesicles; MLV, multilamellar vesicles; NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; BHK-21, baby hamster kidney cells; VSV, vesicular stomatitis virus; G protein, VSV glycoprotein; M protein, VSV matrix protein; Tris, tris(hydroxymethyl)-aminomethane.

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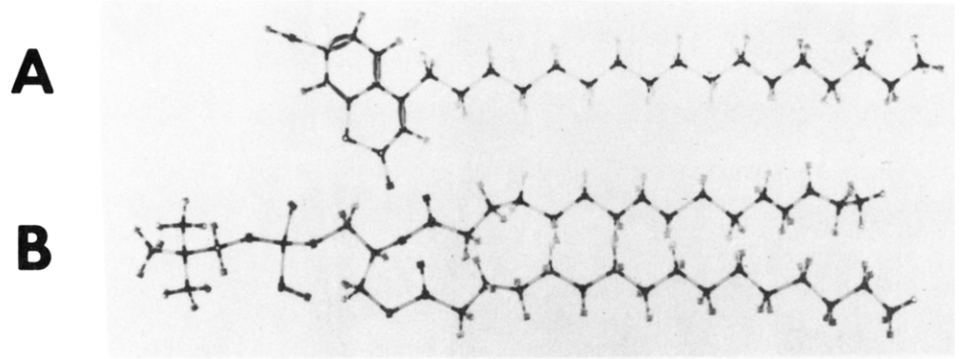

FIGURE 1: Models for (A) 4-heptadecyl-7-hydroxycoumarin and (B) phospholipid.

bilayer (Tilley et al., 1979; Thulborn & Sawyer, 1978; Vincent et al., 1982). *N*-Phenylnaphthylamine has been used as a probe for the ester carbonyl region of the phospholipid acyl chain (Matayoshi & Kleinfeld, 1981). *trans*-Parinaric acid is a naturally occurring fluorescent fatty acid which preferentially partitions into and reports on the gel-state phospholipids (Sklar et al., 1975).

Most of these probes described above have chromophores residing in the hydrocarbon region of the bilayer. The fluorescent phenolic compound umbelliferone, when attached to a long hydrocarbon chain, intercalates into the lipid matrix of the membrane (Fernandez & Fromherz, 1977; Fromherz, 1973). The coumarin moiety of this fluorescent molecule represents the chromophore while its long paraffinic chains resemble the acyl chains of phospholipids. Ionization of the coumarin moiety on the surface of the bilayer has been utilized to calculated the surface electrical potential of model and biological membranes (Fernandez, 1981; Pal et al., 1983a). Due to the presence of the chromophore at the lipid–water interface, the polarization property of 4-heptadecyl-7-hydroxycoumarin can be used to study the organization of lipids near the polar region of the membrane (Figure 1). In this paper, we report on studies of the steady-state fluorescence anisotropy, emission lifetime, and differential polarized phase fluorometry of this probe in isotropic medium, in phospholipid vesicles, and in the membrane of vesicular stomatitis virus. The results presented here demonstrate that the polarization property of the probe in both model and biological membranes can be utilized to study the organization of lipids near the lipid–water interface.

Experimental Procedures

Materials

Lipids and Fluorescent Probes. 1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) and 1,2-dipalmitoyl-phosphatidylglycerol (DPPG) were obtained from Avanti Biochemicals, Birmingham, AL, while 1,2-dimyristoyl-3-*sn*-phosphatidylcholine (DMPC) was obtained from Sigma Chemical Co., St. Louis, MO. Each lipid stock yielded a single spot on silica gel thin-layer chromatography plates (E. Merck, Darmstadt, West Germany) run in a chloroform/methanol/ammonia (65:25:4) solvent system. 4-Heptadecyl-7-hydroxycoumarin (HC) was obtained from Molecular Probes, Junction City, OR. The probe was dissolved in tetrahydrofuran (10 mM) and was stored at −70 °C in the dark until used.

Cells and Virus. Baby hamster kidney cells (BHK-21) 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., 1976a). Plaque-purified vesicular stomatitis virus (VSV) 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 21-h postinfection and were purified by differential, rate zonal, and equilibrium centrifugation (Barenholz et al., 1976a). Purified virions were stored in phosphate buffer (pH 7.4) at a concentration of 2–5 mg/mL at −70 °C until further use.

Methods

Preparation of Lipid Vesicles. Small unilamellar lipid vesicles (SUV) were prepared by sonication as previously described (Barenholz et al., 1977). Various lyophilized lipids were resuspended in 3–4 mL of the appropriate buffer and sonicated under a nitrogen atmosphere at different temperatures depending on the lipid composition. The suspensions were then centrifuged at 100000*g* for 90 min to pellet residual multilamellar vesicles. The supernatant unilamellar vesicles were removed and used immediately.

Lipid and Protein Analysis. The phospholipid content of vesicles 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).

Reconstitution of the Glycoprotein (G) and Matrix Protein (M) of VSV into Lipid Vesicles. (A) G Protein. The glycoprotein was isolated from VS virions by solubilization with octyl glucoside and reconstituted into DPPC vesicles by the detergent dialysis method as described elsewhere (Petri & Wagner, 1979).

(B) M Protein. The matrix protein was isolated from VS virions by solubilizing the virions in 10 mM Tris (pH 8.0) containing 0.25 M NaCl and 1% Triton X-100 and purified by using phosphocellulose column chromatography (Zakowski & Wagner, 1980). The pure protein was reconstituted into performed DPPC/DPPG small unilamellar vesicles as described by Wiener et al. (1983).

Depletion and Enrichment of Cholesterol in the Membrane of VSV. Cholesterol was depleted from the viral membrane by incubating virions for 16 h with serum enriched with sphingomyelin (SPM) (Pal et al., 1981). Nearly 70% of cholesterol from the viral membrane was depleted after such treatment. For cholesterol enrichment, virions were incubated for 10 h with serum loaded with cholesterol (Pal et al., 1980). Cholesterol content in the VSV membrane was increased from the normal 35 mol % to 45 mol % after such enrichment. The virions after such treatment were purified by sucrose density gradient centrifugation as described elsewhere (Pal et al., 1981).

Fluorescence Techniques. (A) Steady-State Fluorescence Anisotropy. Steady-state fluorescence anisotropy was per-

formed exactly as described by Barenholz et al. (1976a) using a modified Perkin-Elmer MPF3 spectrofluorometer with polarizers in the excitation and emission beams. When fluorescence depolarization was measured as a function of temperature, the sample was heated to 55 °C and then cooled at a rate of 25 °C per h. The correction due to scattered light was applied as described before (Barenholz et al., 1976a). The probe was excited at 325 or 375 nm, and the steady-state anisotropy was measured at 450 nm by using a 41 filter.

(*B*) *Emission Lifetime.* The emission lifetime was measured directly in an updated version of the cross-correlation phase and modulation fluorometer from SLM Instruments (Urbana, IL). The excitation wavelength 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-Bebye ultrasonic-light modulator. The lifetime was measured by the phase shift 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 continuous until stable values were obtained.

(*C*) *Differential Phase Fluorescence Measurements.* 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 the emission lifetime measurement. The nonzero limiting anisotropy (r_∞) at a time long compared to the fluorescence lifetime was calculated by the combined use of lifetime, differential lifetime, and steady-state fluorescence anisotropy measurements as described elsewhere (Lakowicz et al., 1979; Lakowicz, 1981).

Differential Scanning Calorimetry (DSC). DSC measurements were performed by using an instrument similar to that previously described (Suurkuusk et al., 1976; Barenholz et al., 1976b). This instrument was modified to work also in a cooling mode as well as in the previously described heating mode. Both heating and cooling rates were controlled by a PDP 11/10 computer attached to the calorimeter, having a maximal cooling rate of −9 °C/h (due to the capacity of the cooling coils). All experiments were performed at a constant rate of 10 °C/h for the heating mode and −7 °C/h for the cooling mode.

NMR Measurements. Proton NMR measurements were performed by using a Bruker WH 300 pulse Fourier transform spectrometer. The desired temperature (±0.5 °C) was obtained by using the Bruker temperature control unit. The instrument was locked on the D_2O reference. Typical conditions were a spectral width of 3.2 kHz with 4K data points in the frequency domain. Excellent signal to noise ratios were obtained for 10 mM phospholipids after 80–150 transients were averaged by using a recycle time of 2 s (identical spectra were obtained by using a recycle time of 5 s but not after 1 s). A pulse width of 1.5 μs was used. The relative number of protons was determined from the area of the separated resonances by the integral height and by planimetry using the Apple II graphic tablet system.

Results and Discussion

4-Heptadecyl-7-hydroxycoumarin (HC) is a weak acid and as such can be present in unionized or ionized states. The unionized state of the probe excites at 325 nm and has a weak emission at 390 nm. The ionized form of the molecule has a much larger quantum yield than the unionized species and has excitation and emission maxima at 375 and 450 nm, respectively. The ratio between these two forms can be determined from the fluorescence excitation and emission spectra of this fluorophore (Fernandez, 1981; Pal et al., 1983a). The degree of ionization of the probe in model and biological membranes is dependent upon the local pH which, together with the fact that HC is amphipathic, having a long paraffinic chain and a polar head group, has made it a suitable candidate to determine the electrical surface potential of membranes (Fernandez, 1981; Pal et al., 1983a). As the chromophore of the probe resides in the bilayer at the lipid–water interface, studies on fluorescence depolarization of HC should help in understanding the structural properties of the head-group region of phospholipids in the membrane. In this paper, we have investigated extensively the steady-state fluorescence anisotropy, emission lifetime, and differential polarized phase fluorometric behavior of HC in isotropic viscous glycerol medium, in phospholipid vesicles, and in the membrane of VSV. Since fluorophores may impose either local or general perturbations on lipid bilayers, we have also employed NMR spectroscopy and differential scanning colorimetry to study the effect of the probe on the organization of lipid bilayers in phospholipid vesicles and compared these results with those of vesicles containing DPH.

Table I: Lifetime (γ), Differential Tangent (tan Δ), and Steady-State Fluorescence Anisotropy (r_s) of 4-Heptadecyl-7-hydroxycoumarin in Glycerol Solution[a]

temp (°C)	γ (ns)	tan Δ	r_s
45	4.85	0.167 (0.179)[b]	0.148
40	4.88	0.163	0.178
35	4.94	0.153	0.206
30	4.89	0.132	0.234
25	4.86	0.105	0.265
20	4.85	0.083	0.289

[a] Ten nanomoles of HC was added to 3 mL of glycerol solution. The probe was ionized completely by raising the pH of the glycerol solution with 5 μL of 1 N NaOH. The lifetime, differential tangent, and steady-state fluorescence anisotropy were measured as described in the text. [b] $\tan \Delta_{max}$ calculated theoretically as described by Lakowicz et al. (1979).

Isotropic Viscous Medium as a Standard for Studying Polarization of 4-Heptadecyl-7-hydroxycoumarin. Glycerol was selected as the isotropic viscous medium because its polarity is high enough to resemble a lipid–water interface. The pH of the glycerol solution was varied by adding minimal amounts of a concentrated NaOH solution. The excitation and emission spectra of the probe in glycerol were affected by the pH in the medium. The anionic form of the probe has a higher quantum yield than did the unionized form. For measurement of the fluorescence anisotropy and lifetime of the fluorophore in the glycerol medium, 10 nmol of the probe in tetrahydrofuran was added to 3 mL of pure glycerol solution (Sigma Chemical Co., St. Louis, MO), and the pH of the glycerol was increased to 9.0 by adding NaOH.

(*A*) *Steady-State Anisotropy and Lifetime.* As presented in Table I, the steady-state fluorescence anisotropy of the probe in glycerol was found to increase with a decrease in temperature. The lifetime of the probe in glycerol was also determined as a function of temperature, and some of the values are presented in Table I. The lifetime of the fluorophore in glycerol for the temperature range of 10–50 °C was constant and was independent of temperature (4.88 ± 0.03 ns). Perrin plots are important parameters to describe the relationships between fluorescence depolarization of a fluorophore in isotropic medium and the medium viscosity. The limiting anisotropy (r_0) of HC as required for the Perrin plot was obtained

by determining the fluorescence anisotropy of the probe in propylene glycol at −60 °C. Under this condition, propylene glycol is present in a glasslike structure of very high viscosity (Shinitzky & Barenholz, 1974). The r_0 value obtained under such conditions was 0.367 ± 0.005, which indicates that the absorption and emission dipoles are almost colinear.

(*B*) *Perrin Plot.* The Perrin plot can be described as follows:

$$\frac{r_0}{r} = 1 + \frac{R_g T \tau}{V \eta}$$

in which r_0 and r are the limiting anisotropy and the observed steady-state anisotropy, respectively, R_g is the gas constant, T is the absolute temperature (in degrees kelvin), τ is the fluorophore lifetime, V is the effective hydrodynamic volume of the rotating fluorophore, and η is the viscosity of the medium (Shinitzky & Barenholz, 1974). A linear Perrin plot was obtained for HC in glycerol with an intercept (r_0/r) of 1.06 ± 0.06 at infinite viscosity. The effective volume of HC in glycerol obtained from the slope of the Perrin plot was 192 Å^3, which was unaffected by the viscosity of the medium. Incidently, a nonlinear Perrin plot was obtained for probes like DPH in various viscous media (Shinitzky & Barenholz, 1978).

To determine whether the depolarizing rotation of 4-heptadecyl-7-hydroxycoumarin was hindered in glycerol, the differential tangent of the probe in glycerol was determined. The results in Table I show that the differential tangent varied greatly with temperature, showing a maximum value at 45 °C and gradually decreasing at lower temperature. The maximum differential tangent (tan Δ_{max}) was calculated theoretically as described by Lakowicz et al. (1979) and was found to be in close agreement with the observed value. This close agreement between the observed and theoretically calculated differential tangents suggests that, in glycerol, HC is a free isotropic rotator.

All these studies clearly demonstrate that 4-heptadecyl-7-hydroxycoumarin undergoes depolarization in glycerol medium and the viscosity of the medium has a large effect on its steady-state anisotropy. The probe appears to have a relatively short lifetime which is unaffected by temperature. The depolarizing rotations of the fluorophore in glycerol solution are isotropic and unhindered.

Polarization of 4-Heptadecyl-7-hydroxycoumarin in Phospholipid Vesicles. Depolarization of HC in phospholipid vesicles was studied in order to establish whether the probe can be used to study the organization of bilayers near the lipid–water interface of the membrane. Before such depolarization behavior was studied, it was necessary to establish whether the presence of probe in the membrane has any perturbing effect on the bilayer. The interaction of HC with the lipid bilayer was determined by differential scanning calorimetry (DSC) and NMR spectroscopy.

(*A*) *Differential Scanning Calorimetry.* For studying the effect of HC and the thermotropic behavior of lipids, DPPC multilamellar vesicles (MLV) containing 1–2 mol % HC were prepared in 0.05 M KCl, and the vesicles were scanned in the heating mode. The thermogram scans of DPPC MLV containing the probe were found to be identical with those obtained from pure DPPC vesicles (data not shown). The main gel to liquid-crystalline phase transition temperature (T_m) was 41.69 °C for pure DPPC vesicles while it was 41.76 °C for DPPC vesicles containing 1.5 mol % HC. The $\Delta t_{1/2}$ and ΔH values for DPPC MLV were 0.6 °C and 8.46 kcal/mol, respectively, while the values were 0.65 °C and 8.43 kcal/mol, respectively, for DPPC MLV containing 1.5 mol % HC. The pretransition of DPPC MLV was found to be almost identical with that of MLV containing 1.5 mol % HC.

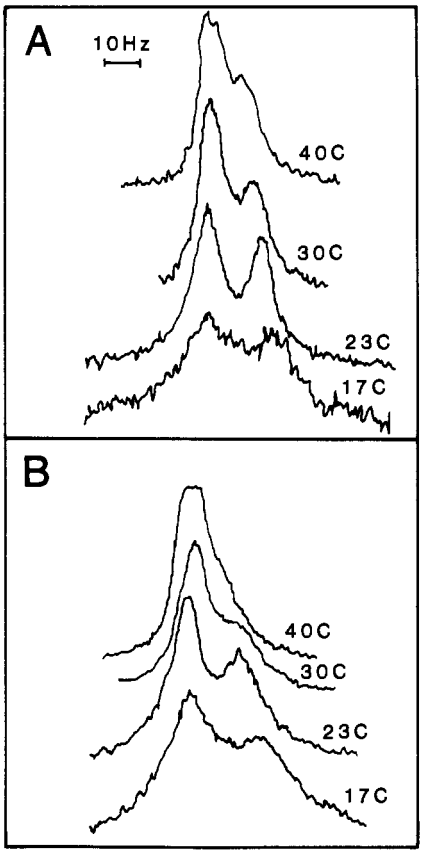

FIGURE 2: 300-MHz NMR spectra of DMPC vesicles containing 1 mol % 4-heptadecyl-7-hydroxycoumarin. DMPC small unilamellar vesicles were prepared by colyophilizing the probe with DMPC prior to vesicle preparation. NMR spectra were recorded at different temperatures. $-N^+(CH_3)_3$ protons of (A) DMPC vesicles alone and (B) DMPC vesicles containing 1 mol % HC.

From this study, it is clear that 4-heptadecyl-7-hydroxycoumarin does not have a large overall effect on lipid organization nor does it perturb the bilayer significantly. It should be noted that DPH, like HC, has almost no effect on the DPPC main transition although some effect was detected on the $\Delta t_{1/2}$ values (Y. Barenholz, unpublished results). *trans*-Parinaric acid, on the other hand, has a rather pronounced effect on the DPPC MLV main transition, shifting it to the higher temperature and causing the transition pattern to be very asymmetrical. This may be due to the preferential partitioning of *trans*-parinaric acid into the gel phase of the membrane (Y. Barenholz, unpublished results).

(*B*) *Proton NMR Spectroscopy.* The effect of HC on the organization of the lipid bilayer was also examined by ^1H NMR spectroscopy. For this experiment, dimyristoylphosphatidylcholine (DMPC) small unilamellar vesicles containing 1.0 mol % HC were prepared by colyophilizing the probe with DMPC prior to preparation of sonicated unilamellar vesicles (SUV). Due to the high resolution power of the Bruker 300-MHz NMR spectrometer, no shift reagent was required to obtain the separation of inside to outside choline signals. As shown in Figure 2, the inside/outside separation of choline signals ($\Delta\delta_H$) was dependent on temperature. Below the phase transition temperature, chemical shift differences between the inside to outside choline signals ($\Delta\delta_H$) were unaffected by the presence of HC in the vesicles. Thus, at 17 °C, the value of such a chemical shift difference was 19.95 Hz for DMPC vesicles alone, while it was 20.34 Hz for DMPC vesicles containing 1 mol % HC. However, above the phase transition temperature, much smaller separation of two choline

signals was obtained in the presence of HC. Thus, at 40 °C, the chemical shift difference between inside/outside choline was 12.13 for DMPC vesicles, but no separation of choline signals was observed in DMPC vesicles containing 1 mol % HC. This effect was shown to increase with an increase in probe content in the bilayer (data not shown). The change in the splitting between the inner and outer choline protons can be explained by the effect of HC on the packing density and orientation of choline head groups in the membrane. The fluorophore HC has a long paraffinic chain which can be inserted between the acyl chains of phospholipid molecules forming the bilayer (see Figure 1). The hydrocarbon chain of the probe will have but a minimal effect on the organization of the acyl chain in the membrane since the polar coumarin moiety of the fluorophore would force the paraffinic chain to lie parallel to the fatty acyl chains of phospholipids in the bilayer. However, HC has a rather large head group (coumarin moiety) which is located parallel to the head group of the phosphatidylcholine moiety in the membrane (Figure 1). Since the inside to outside separation of the choline signal was reduced considerably in vesicles containing the probe, it is quite likely that the packing density and orientation of the head groups in the two monolayers have become more alike. If the vesicle size does not change in the presence of the probe, this can be explained by the effect of the fluorophore on either the outer leaflet of the membrane, making it pack more densely, or the inner leaflet, making it less dense. The effect of incorporation of the probe on the size of the vesicles was ruled out on the basis of the inside/outside choline proton signal intensity ratio, by turbidity measurements, and also from the identical data obtained by incorporation of the probe into preformed DMPC vesicles. These DMPC vesicles were small and seemed to reach their minimal size with larger curvature in the inner monolayer than in the outer monolayer. Therefore, further change in the packing density of the inner monolayer seems difficult.

This leads us to assume that the major effect of HC following incorporation in the membrane is on the outer leaflet of the bilayer. A similar effect on the packing density of the head groups of phospholipids was also observed in distearoylphosphatidylcholine vesicles during the phase transition of lipids in the membrane (Eigenberg & Chan, 1980). All these experiments support the evidence that HC affects the packing density of the head-group region of the bilayer, presumably on the outer monolayer, and has almost no effect on the acyl chains of the phospholipids in the membrane. This effect will be more pronounced in highly curved bilayers.

(*C*) *Fluorescence Polarization Study.* The fluorescence depolarization behavior of HC in DPPC and DMPC small unilamellar vesicles was determined by measuring the steady-state (r_s) and limiting anisotropy (r_∞) of the probe embedded in the bilayer of the vesicles. For comparison, the steady-state anisotropy of DPH and TMA-DPH in DPPC vesicles was also measured. As shown in Figure 3, the steady-state fluorescence anisotropy of HC showed a large change during the phase transition of DPPC and DMPC vesicles. In contrast to the sharp phase transition detected by DPH and TMA-DPH, the change shown by HC was rather broad. Further, the anisotropy values (r_s) in the gel state for HC were significantly lower than those for DPH and TMA-DPH in DPPC vesicles. In the liquid-crystalline state, the steady-state anisotropy values were comparable for DPH and HC in DPPC vesicles, although the values were significantly lower than those shown by TMA-DPH. The limiting anisotropy (r_∞) values of HC also showed a marked change

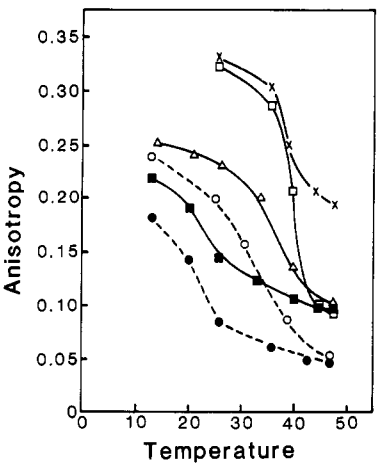

FIGURE 3: Steady-state (r_s) and limiting anisotropy (r_∞) of 4-heptadecyl-7-hydroxycoumarin in DPPC and DMPC vesicles as a function of temperature. Limiting anisotropy (r_∞) at different temperatures was calculated from differential tangents, lifetime, and steady-state anisotropy, as described by Lakowicz et al. (1979). 500 nmol of vesicles in 10 mM Tris-HCl buffer (pH 8.9) was mixed with 2.5 mol of probe at 45 °C. The mixture was incubated at 45 °C for 2 h, and the steady-state anisotropy was measured. DPH and TMA-DPH probes were incorporated into DPPC small unilamellar vesicles in a similar fashion. Steady-state fluorescence anisotropy of HC in (△) DPPC vesicles and (■) DMPC vesicles. Limiting anisotropy of HC in (○) DPPC and (●) DMPC vesicles. Steady-state anisotropy of DPH in DPPC (□) and of TMA-DPH in DPPC (×) vesicles.

during the phase transition of DMPC and DPPC lipids. The limiting anisotropy contributed a significant fraction to the steady-state anisotropy in the gel state compared to the liquid-crystalline state. Thus, at 20 °C for DPPC vesicles, the r_∞ value was 0.22 as compared to 0.24 for steady-state anisotropy, while at 45 °C, r_∞ was 0.05 against a steady-state anisotropy value of 0.11. Indeed, a similar high contribution of r_∞ to steady-state anisotropy was also noted for DPH and TMA-DPH in the gel state of the lipid bilayer, suggesting that the measured fluorescence anisotropy of a probe in the gel state mostly reflects the structural order of the bilayer. It is to be noted that although the ionization of HC was strongly affected by the pH of the solution (Fernandez, 1981; Pal et al., 1983a), both steady-state and limiting anisotrophy were not significantly affected by the degree of ionization of the probe (data not shown).

(*D*) *Emission Lifetime and Differential Tangent.* The emission lifetime and differential tangent of HC in DPPC and DMPC small unilamellar vesicles were measured as a function of temperature. For the temperature range of 10–50 °C, the lifetime of the probe in both DPPC and DMPC vesicles was independent of the temperature and physical state of the lipids. The average lifetime values were 4.61 ± 0.04 and 4.63 ± 0.07 ns for DPPC and DMPC vesicles, respectively, for the temperature range studied. In this context, it is to be noted that the lifetimes of probes like DPH, TMA-DPH, and *trans*-parinaric acid are strongly influenced by temperature and by the physical state of lipids (Lakowicz, 1981; Prendergast et al., 1981).

The differential tangents of the fluorophore in DPPC and DMPC small unilamellar vesicles are shown in Table II. The differential tangents in both DPPC and DMPC vesicles revealed a steady increase near the phase transition of the lipids and decreased as the temperature was lowered in the gel state. However, as shown in Table II, the differential tangent values for DPH and TMA-DPH were decreased to a large extent in the gel state when compared to HC. This suggests that the

Table II: Comparative Differential Tangents (tan Δ) of HC in DPPC and DMPC SUV and DPH and TMA-DPH in DPPC SUV[a]

temp (°C)	tan Δ (HC) DPPC	tan Δ (HC) DMPC	tan Δ (DPH) DPPC	tan Δ (TMA-DPH) DPPC
46	0.099	0.096	0.136	0.067
42	0.113	0.094	0.137	0.075
39	0.103	0.105	0.073	0.043
33	0.084	0.114	0.035	0.021
25	0.065	0.118	0.024	0.015
20	0.058	0.072		
14	0.050	0.075		

[a] All the vesicle preparations were made in 10 mM Tris-HCl buffer, pH 8.9. The lifetime [γ (in nanoseconds)] of HC in DPPC and DMPC SUV was constant at all temperatures and was 4.61 ± 0.04 and 4.63 ± 0.07 ns for DPPC and DMPC vesicles, respectively.

probe is less restricted in motion especially in the gel state as compared to DPH and TMA-DPH. The theoretically calculated $\tan\Delta_{max}$ of HC in the lipid bilayer was calculated as described by Lakowicz et al. (1979) and was found to be nearly 50% of the experimentally determined value. This shows that the depolarization rotation of HC in the lipid bilayer is hindered as observed for DPH or TMA-DPH (Lakowicz et al., 1979; Prendergast et al., 1981).

The lifetime of HC in fused DPPC unilamellar vesicles (a gift from T. E. Thompson, University of Virginia) was studied at two different pH levels to examine the effect of ionization on the lifetime of the probe. The p*K* of HC in neutral phospholipid vesicles was 8.65, and thus at pH 7.0, most of the HC molecules were unionized while at pH 8.9 these were mostly ionized. For the temperature range of 20–50 °C, the emission lifetime of the probe was constant and was unaffected by the pH (4.72 ± 0.15 ns) although the quantum yield at pH 8.9 was larger than at pH 7.0 (data not shown).

(*E*) *Cholesterol Effect.* The interaction of cholesterol with lipid bilayers is the subject of many investigations (Cooper, 1977; Huang, 1977; Barenholz, 1984). It has been shown that while cholesterol disorders the lipids in the gel state, a significantly ordering effect is observed in the liquid-crystalline state of the membrane lipids (DeKruiff et al., 1972). Using DPH and HC as fluorophores, we examined the effect of incorporation of cholesterol on the order of lipids in two different planes of the bilayer: the head-group plane and the hydrophobic region. To this end, the steady-state fluorescence anisotropy of HC and DPH was measured as a function of temperature in DPPC MLV containing either no cholesterol or 25 mol % cholesterol. The results are shown in Figure 4. At this level of cholesterol in the bilayer, no phase transition of the lipids could be detected by differential scanning calorimetry (Estep et al., 1978). Using both HC and DPH, we observed that the phase transition of DPPC was very broad in vesicles containing cholesterol. In the gel state of bilayer lipids, significant disordering effects by cholesterol were exhibited by both HC and DPH. On the other hand, a marked ordering effect was observed in the liquid-crystalline state of the lipids in the presence of cholesterol when DPH was used a probe, whereas no such ordering effect was noted with HC. All these experiments demonstrate that in the liquid-crystalline state, cholesterol orders the bilayer in the hydrocarbon region with little effect on the head-group region while in the gel state both regions of the bilayer are disordered. Thus, the information on the dynamics of lipids obtained by using these two probes is much more complete than can be obtained by each of the probes separately.

Differential Polarized Phase Fluorometric Behavior of HC in Proteoliposomes Reconstituted with the Glycoprotein or Matrix Protein of VSV. As mentioned before, the VSV

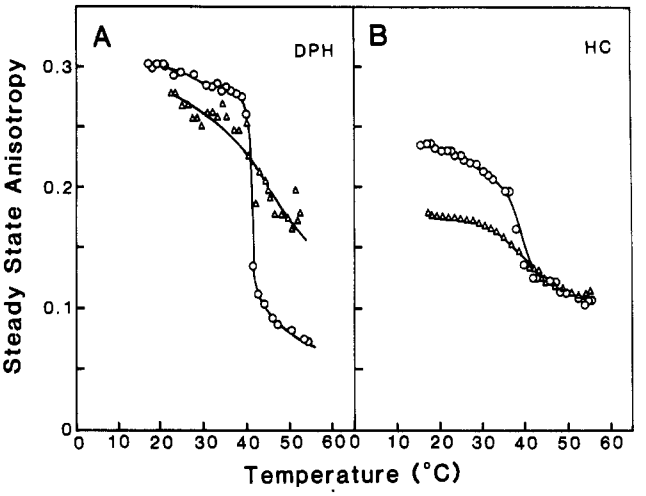

FIGURE 4: Steady-state fluorescence anisotropy as a function of temperature of (A) HC and (B) DPH in DPPC SUV containing no cholesterol (○) or 25 mol % cholesterol (△).

membrane has two proteins. The glycoprotein (G) is an integral membrane protein while the peripheral matrix protein (M) lines the inner surface of the viral membrane (Schloemer & Wagner, 1975; Zakowski & Wagner, 1980). Recent studies in our laboratory have demonstrated that both G and M proteins of VSV could be reconstituted into phospholipid vesicles to form proteoliposomes (Petri & Wagner, 1979; Zakowski et al., 1981). These reconstituted lipid vesicles have provided useful models to study lipid–protein interactions in the bilayer (Petri et al., 1980, 1981; Pal et al., 1983b; Weiner et al., 1983). In this section, we report studies on the effect of G and M proteins on the dynamics of lipids near the polar region of the membrane as monitored by HC. The glycoprotein was reconstituted into DPPC vesicles by the detergent dialysis method as described by Petri & Wagner (1979) while the M protein was reconstituted into DPPC/DPPG sonicated vesicles as described elsewhere (Wiener et al., 1983). Reconstitution of M protein required the presence of a negatively charged phospholipid in the vesicles while no such requirement was noted for G protein.

Differential polarized phase fluorometric techniques were used to determine the interaction of G and M proteins with the lipid bilayer. The emission lifetime of HC in DPPC and DPPC/DPPG vesicles with or without the G or the M protein, respectively, was unaffected by temperature or by the presence of protein in the bilayer. In DPPC vesicles, the lifetime was 4.27 ± 0.05 or 4.37 ± 0.11 ns with or without the G protein, respectively, while in DPPC/DPPG vesicles it was 4.3 ± 0.12 or 4.47 ± 0.038 ns with or without the M protein, respectively. This suggests that no energy transfer had taken place between the tryptophan residues of the proteins and the fluorophore in the membrane. It should be noted that the emission lifetime of DPH and TMA-DPH was affected by the presence of 0.5 mol % of G or M in the lipid bilayer (Wiener et al., 1983; Pal et al., 1983b).

The differential tangent (tan Δ) of the fluorophore observed in vesicles reconstituted with or without G protein is shown in Table III. The insertion of G protein in DPPC vesicles had little effect on tan Δ above 39 °C in the liquid-crystalline state but increased the tan Δ value significantly in the gel state (Table III). The M protein, on the other hand, had little effect on tan Δ above 43.7 °C in the liquid-crystalline state but decreased the tan Δ in the gel state (Table IV).

The limiting anisotropy (r_∞) values of HC in the lipid vesicles with or without G or M protein were calculated by

Table III: Differential Tangent (tan Δ) and Limiting Anisotropy (r_∞) of HC in Lipid Vesicles Reconstituted with G Protein of VSV as a Function of Temperature[a]

temp (°C)	tan Δ		r_∞	
	DPPC	DPPC + G	DPPC	DPPC + G
45.0	0.104	0.109	0.06	0.06
43.0	0.101	0.105	0.07	0.06
39.0	0.081	0.099	0.15	0.10
37.5	0.075	0.083	0.16	0.14
35.0	0.062	0.081	0.19	0.17

[a] The G protein was reconstituted into DPPC vesicles by the detergent dialysis method (Petri & Wagner, 1979). 300 nmol of vesicles containing either no protein or 0.5 mol % protein was resuspended in 10 mM Tris-HCl buffer, pH 8.9, and 1.5 nmol of HC was incorporated into the vesicles at 50 °C. Lifetime (γ) and differential tangent were measured at different temperatures as described under Experimental Procedures while the limiting anisotropy was calculated from lifetime, differential tangent, and steady-state anisotropy, as described by Lakowicz et al. (1979). Lifetime (γ) was found to be constant at all temperatures, and it was 4.37 ± 0.11 and 4.27 ± 0.05 ns in DPPC vesicles without or with protein, respectively.

Table IV: Differential Tangent (tan Δ) and Limiting Anisotropy (r_∞) of HC in Lipid Vesicles Reconstituted with M Protein of VSV as a Function of Temperature[a]

temp (°C)	tan Δ		r_∞	
	DPPC/ DPPG	DPPC/ DPPG + M	DPPC/ DPPG	DPPC/ DPPG + M
51.0	0.0904	0.104	0.06	0.05
47.0	0.101	0.105	0.06	0.07
43.0	0.105	0.075	0.05	0.16
42.0	0.105	0.065	0.07	0.19
37.0	0.088	0.058	0.13	0.22

[a] The M protein was reconstituted into DPPC/DPPG vesicles as described elsewhere (Wiener et al., 1983). 300 nmol of vesicles, containing either no protein or 0.5 mol % protein, was resuspended in 10 mM Tris-HCl buffer, pH 8.9, and 1.5 nmol of HC was incorporated into the vesicles at 50 °C. Lifetime was found to be constant at all temperatures, and it was 4.47 ± 0.038 and 4.3 ± 0.12 ns in vesicles without or with protein, respectively.

the combined use of steady-state anisotropy, emission lifetime, and differential tangents as described by Lakowicz et al. (1979), and the results are also shown in Tables III and IV. Above 40 °C, the G protein had little effect on the limiting anisotropy of HC in the liquid-crystalline phase, while below 39 °C the limiting anisotropy and thus the lipid order parameter were significantly reduced in the presence of the G protein. This effect of the G protein on the lipid bilayer is similar to that of cholesterol as monitored by HC (Figure 4). Indeed, similar results were obtained with DPH and TMA-DPH as fluorophores (Pal et al., 1983b). In DPPC/DPPG vesicles containing M protein, the limiting anisotropy and thus the lipid order parameter were not affected in the liquid-crystalline state, but below 47 °C, the protein increased the limiting anisotropy markedly, leading to increased structural order of the lipid bilayer. An increase of 6 °C in the phase transition temperature was observed in M protein reconstituted lipid vesicles. Indeed, a similar ordering of the gel-state lipids and an increase in the phase transition temperature were observed in lipid vesicles reconstituted with VSV M protein by using DPH and TMA-DPH as fluorescent probes (Wiener et al., 1983).

The disordering effect of G protein in the gel state of the membrane is consistent with the effect of integral membrane proteins in other systems (Chapman, 1982). The M protein, on the other hand, binds to the lipid bilayer and behaves like a peripheral membrane protein by interacting with negatively charged phosphatidylglycerol residues; the M protein also increased the transition temperature and the order of the lipid in the gel state, presumably by inducing phase separation in the plane of the bilayer. Preliminary studies using pyrene lipid analogues in M protein reconstituted vesicles support this assumption (J. R. Wiener et al., unpublished results). These experiments clearly demonstrate that, like other fluorescent probes, HC can be utilized to study lipid–protein interactions in model and biological membranes. The localization of HC near the head groups of the phospholipids and the fact that the lifetime of the probe is unaffected by temperature, by the physical state of lipids, or by the presence of integral or peripheral proteins can serve as a useful probe to study the interaction of membrane protein with lipid bilayer in many complex systems.

Fluorescence Depolarization of 4-Heptadecyl-7-hydroxycoumarin in the VSV Membrane. Various techniques of fluorescence spectroscopy have been successfully used to study the organization and dynamics of biological membranes (Yguerabide, 1981; Lakowicz, 1981). In this section, we describe the fluorescence depolarization and emission lifetime of HC in the membrane of the intact vesicular stomatitis virion. Most of the fluorophores previously used to study the membrane of VSV, such as DPH, TMA-DPH, and *trans*-parinaric acid (Barenholz et al., 1976; Petri et al., 1981; Pal et al., 1983b), monitor the hydrophobic region of the bilayer. Vesicular stomatitis virions consist of a nucleocapside core and a limiting membrane derived from the plasma membrane of infected host cells (Patzer et al., 1979; Wagner, 1975). The viral membrane has two proteins. The externally oriented glycoprotein (G) 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 in close proximity to the nucleocapside (Schloemer & Wagner, 1975; Zakowski & Wagner, 1980; Pal et al., 1981). The lipid composition of the virus is well-defined and is, for the most part, a reflection of the lipid composition of the host cell plasma membrane (Patzer et al., 1978). Cholesterol and phospholipids are the two major lipid components in the envelope, and both of them have been shown to distribute asymmetrically in the bilayer (Patzer et al., 1978; Pal et al., 1981).

(A) Steady-State Fluorescence Anisotropy of HC in VSV Membrane. The incorporation of HC in viral membrane was achieved by adding the probe in tetrahydrofuran to a VSV suspension at a ratio of 1 probe to 100 lipid molecules. The viral suspension was incubated for 3 h at 37 °C and pelleted by centrifugation at 60000*g* for 80 min through a glycerol pad to remove unbound probe. The virion pellet was resuspended in 50 mM KCl. Although the final concentration of HC in the virion membrane varied between experiments, it was always lower than 1 mol % of the total viral lipids. The fluorophore in the viral membrane was ionized by adding NaOH to bring the pH of the viral suspension to 9.5, and the steady-state fluorescence anisotropy was measured at different temperatures. As shown in Figure 5, the steady-state fluorescence anisotropy of the fluorophore was found to increase with decreasing temperature.

(B) Cholesterol Effect. We have previously demonstrated, using DPH as a fluorophore, that depletion of cholesterol from the viral envelope decreased the structural order of lipids in the membrane (Pal et al., 1983b). As shown in Figure 5, cholesterol enrichment of the membrane of VSV did not affect the steady-state fluorescence anisotropy of HC in the viral membrane except at lower temperature, where the anisotropy showed very little increase. On the other hand, depletion of cholesterol from the membrane lowered the steady-state fluorescence anisotropy significantly throughout the range of

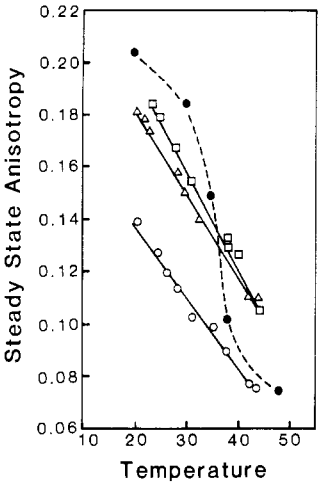

FIGURE 5: Steady-state fluorescence anisotropy of HC in VSV membrane as a function of temperature. The HC probe was incorporated into viral membrane as described in the text. Cholesterol concentration in the envelope of VSV was manipulated as described under Experimental Procedures. Steady-state fluorescence anisotropy was measured in VSV suspension (100 μg/mL) in 0.05 M KCl solution at pH 9.5. Control VSV (△); cholesterol-depleted VSV (○); cholesterol-enriched VSV (□). DPPC fused vesicles (300 nmol) were added to control VSV containing HC and incubated at 37 °C for the steady-state fluorescence anisotropy of mixed DPPC vesicles and virus (●).

temperatures studied. It should be noted that with DPH we observed a similar effect following depletion of cholesterol, but enrichment of cholesterol increased the steady-state fluorescence anisotropy to some extent (Pal et al., 1980, 1981). These experiments thus demonstrate that while depletion of cholesterol from the viral membrane affected the organization of lipids in the polar and hydrocarbon regions of the membrane, enrichment of cholesterol into the viral membrane affected the organization of the hydrophobic region of the bilayer. In this context, it should be noted that with *trans*-parinaric acid a broad phase transition was observed in the membrane of VSV following depletion of cholesterol (Pal et al., 1983b). Thus, it appears that DPH and HC, unlike *trans*-parinaric acid, failed to report the presence of any gel-state lipids because they appeared to distribute uniformly throughout the bilayer.

(*C*) *Lifetime and Differential Tangent of 4-Heptadecyl-7-hydroxycoumarin in VSV Membrane.* The lifetime and differential polarized phase fluorometric behavior of HC in the VSV membrane were studied as a function of temperature. As was true of HC in glycerol and in lipid vesicle systems, the emission lifetime of the probe was found to be independent of temperature in the range of 10–50 °C and had an average value of 5.2 ± 0.05 ns. The observed differential tangent (tan Δ) value gradually decreased when the temperature was lowered below 38 °C. We calculated the theoretical tan Δ_{max} at 45 °C as described by Lakowicz et al. (1979) and observed a large tangent defect because the observed value was nearly 50% that of theoretically calculated value. This suggests that depolarizing rotations of HC in the VSV envelope are highly hindered as observed in lipid vesicles. Indeed, similar hindered rotations of DPH and TMA-DPH were also observed in VSV membrane (Pal et al., 1983b). The limiting anisotropy of HC in VSV membrane was calculated through the combined use of lifetime, differential tangent, and steady-state anisotropy; the values at different temperatures are given in Table V. HC showed a significant value of r_∞ at all temperatures but was greater at lower temperatures. This suggests that the structural order of the VSV membrane increases with a decrease in temperature.

Table V: Differential Tangent (tan Δ) and Limiting Anisotropy (r_∞) of HC in VSV Membrane as a Function of Temperature[a]

temp (°C)	tan Δ	r_∞
45	0.099 (0.195)[b]	0.09
38	0.099	0.10
20	0.077	0.16
10	0.064	0.18

[a] Lifetime (γ) of HC in VSV membrane was constant at all temperatures, and it was 5.21 ± 0.05 ns. [b] tan Δ_{max} theoretically calculated as described by Lakowicz et al. (1979).

Translocation of 4-Heptadecyl-7-hydroxycoumarin between Membranes. To examine whether the HC probe can translocate between various membranes, we used the following approaches which are comparable to those used to study translocation of DPH between membranes (Stubbs et al., 1976; Lentz et al., 1976). In the first approach, we incorporated HC into the membrane of VSV and removed the unbound probe by centrifugation as described under Methods. DPPC SUV were then added to the probe-labeled virions, and the sample was incubated for 30 min at 37 °C. As shown in Figure 5, the steady-state fluorescence anisotropy of mixtures of DPPC vesicles and the virion suspension showed the typical phase transition of DPPC lipids. This can only be explained by the fact that the probe translocated from the virion membrane to the DPPC vesicle bilayer and reported the transition of the lipids. Indeed, similar intermembrane movement of the probe was also noted between vesicles of two different types.

The distribution pattern of HC between the gel and liquid-crystalline phases in the membrane was determined by studying the partitioning of the probe between DMPC and DPPC vesicles. To this end, the fluorophore was incorporated into DMPC and DPPC vesicles separately at a probe to lipid ratio of 1:200, and the steady-state fluorescence anisotropy was measured at 28.5 °C. At this temperature, the DPPC vesicles were in the gel state whereas the DMPC vesicles were in the liquid-crystalline state. The steady-state fluroescence anisotropy of HC in DPPC vesicles was found to be 0.205 at 28.5 °C while that in DMPC vesicles was 0.110. The two preparations of vesicles containing the probe were then mixed at 28.5 °C at equal proportions, and the steady-state fluorescence anisotropy was measured. The resultant anisotropy value was found to be 0.151 (data not shown), very close to the theoretically calculated value of 0.157, which suggests that the probe partitioned equally well in the gel and liquid-crystalline phases of the membrane.

The same results were obtained when similar experiments were conducted in which only one population of vesicles (either DPPC or DMPC) contained HC, which indicated that the fluorophore equilibrated well among vesicles. It is to be noted that DPH also partitioned equally in gel and liquid-crystalline states of lipids (Lentz et al., 1976), while *trans*-parinaric acid has preferential affinity for the gel phase (Sklar et al., 1977).

Conclusions

4-Heptadecyl-7-hydroxycoumarin has been used as a probe to study organization and dynamics of lipids in model and viral membranes. The long hydrocarbon chain of the fluorophore causes its incorporation in the bilayer while the chromophore coumarin moiety resides near the lipid–water interface of the membrane. Thus, the probe serves as a very useful reporter for the polar region of the membrane and can provide much additional information about the lipid organization in model and biological membranes. Such data cannot be obtained by the use of fluorophore residing in the hydrophobic region of the lipid bilayer. However, unlike most of the other fluorescent

probes which reside in the hydrocarbon region of the membrane, HC has a low quantum yield because of its proximity to the bulk aqueous phase outside the membrane. The presence of HC (at least up to 2 mol %) in the bilayer had no effect on the thermotropic behavior of lipids, although the packing density of the head-group regions of the membrane was affected by the probe. The fluorophore detects the phase transition of membrane lipids and reports the order of lipids in the bilayer in the presence of cholesterol and proteins. The probe behaves like a free rotator in glycerol. However, the depolarizing rotations of HC in both model and viral membranes are highly hindered. The unique property of the hydroxycoumarin probe is its very steady lifetime which is unaffected by temperature or by the physical state of lipids. This will help in its application to investigate the organization of many biological and model membrane systems.

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Registry No. HC, 26038-83-5; DPPC, 2644-64-6; DPPG, 4537-77-3; DMPC, 13699-48-4; glycerol, 56-81-5; cholesterol, 57-88-5.

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