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Enveloped Viruses as Model Membrane Systems: Microviscosity of Vesicular Stomatitis Virus and Host Cell Membranes†

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ABSTRACT: The fluorescence probe 1,6-diphenyl-1,3,5-hexatriene was used to study and compare the dynamic properties of the hydrophobic region of vesicular stomatitis virus grown on L-929 cells, plasma membrane of L-929 cells prepared by two different methods, liposomes prepared from virus lipids and plasma membrane lipids, and intact L-929 cells. The rate of penetration of the probe into the hydrophobic region of the lipid bilayer was found to be much faster in the lipid vesicle bilayer as compared with the intact membrane, but in all cases the fluorescence anisotropy was constant with time. The L-cell plasma membranes, the vesicles prepared from the lipids derived from the plasma membranes, and intact cells are found to have much lower microviscosity values than the virus or virus lipid vesicles throughout a wide range of temperatures. The microviscosity of plasma membrane and plasma membrane lipid vesicles was found to depend on the procedure for plasma membrane preparation as the membranes prepared by different methods had different microviscosities. The intact virus and liposomes prepared from the virus lipids were found to have very similar microviscosity values. Plasma membrane and liposomes prepared from plasma membrane lipids also had similar microviscosity values. Factors affecting microviscosity in natural membranes and artificially mixed lipid membranes are discussed.

Enveloped viruses potentially provide simple, controllable model systems for studying membrane composition and function for the following reasons: (i) enveloped viruses bud from surfaces of infected cells and can be readily purified free of cell membranes; (ii) membranes of enveloped viruses appear to be homogeneous; (iii) viral membrane lipids are selected from preformed lipids of host cell membranes, although the lipid proportions differ for the virus and the cell; (iv) viral membranes contain very few protein species, all of which are coded for by the viral genome and not by the cell genome; and (v) the virus genomes do not code for lipid synthesis but evidence is available that the viral membrane proteins select cell lipids for viral membrane assembly by as yet undetermined mechanisms (Lenard and Compans, 1974; Blough and Tiffany, 1973; Choppin et al., 1971).

The purpose of the study described here was threefold: (1) to study some membrane properties of an enveloped virus system, and to investigate the relationship between the host cell plasma membrane and the virus membrane; (2) to describe the advantages and difficulties of using the technique of fluorescence polarization to measure microviscosities of biological membranes; and (3) to compare the results obtained by the fluorescence polarization technique with those obtained with other physical methods such as electron spin resonance (ESR)[1] (Landsberger et al., 1971–1973) and nuclear magnetic resonance (NMR) (Stoffel and Bister, 1975).

1,6-diphenyl-1,3,5-hexatriene was the fluorescence probe used in this study to measure the microviscosities of the hydrophobic regions of viral and host cell membranes. The same probe was used by Shinitzky and Inbar (1974) and Fuchs et al. (1975) to measure the microviscosity of the membranes of lymphocytes and 3T3 cells, respectively. Vesicular stomatitis (VS) virus grown on L cells was selected for this study because the lipid composition of both the virus membrane and the host cell plasma membrane have been described in detail (McSharry and Wagner, 1971). Furthermore the virus has only two proteins in its lipid membrane and these have been well characterized (Wagner et al., 1972b; Schloemer and Wagner, 1975; Moore et al., 1974; Bishop et al., 1975), making this system one of the simplest naturally occurring membranes.

Materials and Methods

Cells. BHK-21 cells were grown as monolayers at 37 °C in 20 ml of BHK-21 medium supplemented with 10% tryptose phosphate broth and 10% fetal calf serum and containing 1%

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[1] Abbreviations used are: VS, vesicular stomatitis; BHK, baby hamster kidney; BME, Basal Medium Eagle; EBSS, Earle's balanced salt solution; PBS, phosphate-buffered saline; RSB, reticulocyte standard buffer; SPM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DPH, 1,6-diphenyl-1,3,5-hexatriene; FMA, fluorescein mercuric acetate; B, bullet; ESR, electron spin resonance; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

each of penicillin and streptomycin. Cells were passed at confluency 1 to 10 in 75-cm^2 surface Falcon plastic flasks and reached confluency within 3 days (about 4×10^7 cells/flask).

L-929 cells were also grown as monolayers at 37 °C in 20 ml of Basal Medium Eagle (BME) containing Hank's salts supplemented with 10% calf serum and 1% each of penicillin and streptomycin. Cells were passed at confluency 1 to 3 in 75-cm^2 surface Falcon plastic flasks and reached confluency within 3 days (about 2×10^7 cells/flask).

Virus Growth and Purification. The Indiana strain of VS virus was cloned by plaque purification and used to infect confluent monolayers of L-929 and BHK-21 cells at a multiplicity of 0.1–1.0 plaque forming units/cell resulting in the formation of infectious B[1] virions. Virus was adsorbed to the monolayers for 1 h at room temperature before overlaying each monolayer with 6 ml of BME in the case of L cells and 6 ml of BHK-21 medium for BHK cells. Labeled virus was produced by introducing 1 μCi/ml of ^{14}C-labeled amino acid hydrolysate (57 mCi/matom) or 5 μCi/ml of [^{3}H]leucine (60 Ci/mmol) and/or [^{3}H]tyrosine (44 Ci/mmol).

The supernatant fluids were collected 16–18 h postinfection and the pooled supernatants were centrifuged at 900*g* for 20 min at 4 °C. All subsequent steps were performed at 4 °C or on ice. The supernatant fluid from this step was then centrifuged at 80 000*g* for 90 min through a 2-ml pad of 50% glycerol in Earle's balanced salt solution (EBSS)[1] in an SW27 rotor. The resulting pellet was gently resuspended into EBSS, layered over a preformed 0–40% sucrose gradient containing 50 mM Tris, 0.25 M NaCl, and 0.5 mM EDTA (pH 7.6) and centrifuged for 90 min at 35 000*g* in an SW25.1 rotor. The clearly visible band of B virions was harvested, diluted approximately fivefold with phosphate-buffered saline (PBS), and pelleted through a 2-ml 50% pad of glycerol in PBS at 60 000*g* for 90 min in an SW25.1 rotor. The virus pellets were drained and gently resuspended in PBS, and the suspension was overlaid on a gradient of 0–40% potassium tartrate to 0–20% glycerol containing 20 mM Tris (pH 7.6). Centrifugation to equilibrium was for 18 h at 35 000*g* in an SW25.1 rotor, and the visible band was harvested, diluted, and pelleted as before. The virus pellets were drained and carefully washed with PBS before suspension in PBS at a concentration of 2–10 mg/ml. Virus was stored at 4 °C for use within 2 days or at −80 °C.

Plasma Membrane Preparation. Method A. The procedure used was essentially the method of flotation equilibrium centrifugation of cell homogenates described by Perdue et al. (1971a,b) for the isolation of plasma membranes from cultured cells. Thirty L-929 monolayers were washed twice with 5 ml of 0.16 M NaCl, suspended by scraping with a rubber policeman, and washed twice more with 40 ml of 0.16 M NaCl. The pelleted cells were suspended in 12 ml of 0.16 M NaCl and disrupted with a Potter-Elvehjem homogenizer until lysis was complete as monitored microscopically. The homogenate was centrifuged at 200 000*g* for 30 min in a SW65 rotor and the pellet was suspended in 85% (w/v) sucrose. A linear gradient of 40–65% (w/v) sucrose and 1 ml of 10% (w/v) sucrose was overlaid over the 85% (w/v) sucrose. The gradient was centrifuged at 60 000*g* in a SW25 rotor for 18 h. The band B near the top of the gradient as described by Perdue et al. (1971a,b) was removed from the gradient, pelleted, and recycled on the gradient described above. Band A, at the top of the gradient, was found to contain a high percentage of free lipid. The visible band was removed, diluted with PBS, and pelleted before resuspension in PBS.

Method B. The procedure used for isolation of plasma membrane was previously described by Wagner et al. (1972a) and is basically the procedure developed by Caliguiri and Tamm (1970). Thirty confluent monolayers of L-929 cells were each washed twice with 5 ml of ice-cold PBS and the cells were suspended by scraping into 5 ml of EBSS. The pooled cells were then pelleted at 800*g* for 10 min, resuspended, and washed twice with EBSS. After a final centrifugation at 1500*g* for 10 min, the cells were drained and resuspended in 15 ml of reticulocyte standard buffer (RSB) containing 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 0.01 M NaCl, 0.0015 M $MgCl_2$ (pH 7.5). The cells were swollen in this buffer for 20 min and then disrupted with 20 strokes in a tightly fitting glass homogenizer. Nuclei and unbroken cells were removed by centrifugation at 800*g* for 10 min. The suspension was then made 30% with respect to sucrose and the following gradient was constructed: 3 ml of 60% sucrose, 7 ml of 45% sucrose, 7 ml of 40% sucrose, 10 ml of sample in 30% sucrose, 7 ml of 25% sucrose, and 3–4 ml of RSB. The gradients were centrifuged at 60 000*g* for 20 h in a SW27 rotor. The top light-scattering band in the region of the 25–30% sucrose interface, previously designated as plasma membrane, was carefully collected, diluted with RSB, and pelleted at 60 000*g* for 90 min in a SW25.1 rotor. The pellets were resuspended in RSB.

Preparation of Lipid Vesicles. Total lipids of both VS virus and plasma membrane were extracted by the method of Folch et al. (1957). The organic solvents were removed by flash evaporation and vesicles were made by high-intensity ultrasonic irradiation of the lipids in PBS for 5 min under nitrogen at 4–10 °C using Heat Systems W-350 sonifier. The clear suspensions obtained by sonication were centrifuged at 100 000*g* for 60 min to remove large liposomes.

Phosphorus Determination. Total phospholipid phosphorus was determined as described by Bartlett (1959).

Cholesterol Determinations. Cholesterol was quantitated, using cholesterol oxidase, by a modification of the procedure of Allain et al. (1974).

Protein Determinations. Protein was determined by the method of Lowry et al. (1951) using crystalline bovine plasma albumin as the standard.

Fluorescence Labeling of the Membrane Fraction. The fluorescence hydrocarbon used as a probe to study the dynamic properties of the hydrophobic region of the biological membranes and lipid vesicles was 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich "Puriss"). Four milliliters of dispersed membrane suspended in PBS was preincubated at 39 °C. One microliter of the desired concentration of DPH in tetrahydrofuran was added to the membrane containing PBS solution. The solution was vigorously mixed for 10 s and then incubated at 39 °C. The penetration of the DPH into the bilayer was followed by measuring the increase in fluorescence intensity (see eq 2) with time. The fluorescence polarization measurements were started when the intensity (F) reached a constant value.

Fluorescence Measurements. Fluorescence polarization of DPH was measured by a method similar to that described by Shinitzky and Barenholz (1974) using a Perkin-Elmer MPF-3 spectrofluorimeter adjusted by us for polarization measurements. All the measurements were done in the ratio mode. Fluorescence intensity and polarization were obtained by measurement of $I_\parallel$ and $I_\perp$ (where $I_\parallel$ and $I_\perp$ are the fluorescence intensities detected through a polarizer oriented parallel ($\parallel$) and perpendicular ($\perp$) to the direction of polarization of the excitation beam). Using a multiwave plate wedge depolarizer (Electrooptics Industry, Rehovot, Israel) between the

analyzer and the detector caused the correction factor for unequal transmission of differently polarized light to be equal to 1.000 as determined by the polarization spectra of Rhodamine B in dry glycerol and *N*-methylacridinium perchlorate in methanol. The ratio of $I_\parallel$ to $I_\perp$ (defined as $A = I_\parallel/I_\perp$) is related to the fluorescence anisotropy (r) by the following equation

$$r = (I_\parallel - I_\perp)/I_\parallel + 2I_\perp = (A - 1)/(A + 2) \quad (1)$$

The r values for DPH are in the range of 0.362 (the limiting anisotropy) for a medium with very high viscosity (propylene glycol at −50 °C) to 0 in a medium with infinitely low viscosity (Shinitzky and Barenholz, 1974). The total fluorescence intensity (F) is described by eq 2

$$F = I_\parallel + 2I_\perp = (A + 2)I_\perp \quad (2)$$

DPH was excited at 360 nm and its fluorescence was detected at 430 nm using the instrument's filter 39 as a cutoff filter for wavelengths below 390 nm. The readings of the fluorescence intensities $I_\parallel$ and $I_\perp$ were monitored by recorder and showed no decrease in intensity during the time of the measurement.

The temperature was controlled using a Lauda MK-2 thermoregulated bath. After the measurement in the highest desired temperature was performed, the temperature of the thermoregulated bath was turned to 0 °C. The temperature change was at an average speed of 3 min per 1 °C. The temperature of the sample was measured continuously in the sample using a Yellow Springs Instrument Co. thermistor and a read-out device. Every 0.5–1 °C, $I_\parallel$, $I_\perp$, and the exact temperature (±0.1 °C) were recorded.

Determination of the Excited State Lifetime. The lifetime was estimated from the fluorescence intensity, as described by Shinitzky and Barenholz (1974). This estimation can be applied to the systems used in this study since the relative quantum yield is similar to that obtained for liposomes made from egg lecithin. This excludes the possibility of quenching of the DPH fluorescence.

Correction for Light Scattering. In most of the samples the contribution of the scattered excitation light to the $I_\parallel$ value was as high as 3–10% at the high temperatures where the intensity is lower. Since the scattering contribution to the $I_\perp$ value is much smaller than to the $I_\parallel$ value, and as the light scattering is not affected by the temperature, all values of $I_\parallel$ and $I_\perp$ have to be corrected for scattering using the following equation (Shinitzky et al., 1971)

$$A = (I_\parallel{}^t - I_\parallel{}^s)/(I_\perp{}^t - I_\perp{}^s) = I_\parallel/I_\perp \quad (3)$$

where $I_\parallel{}^t$ and $I_\perp{}^t$ are the measurable intensities in the presence of the DPH and $I_\parallel{}^s$ and $I_\perp{}^s$ are the values of light scattering obtained for membranes without the DPH. All calculations of anisotropy (r) were done using the corrected value $I_\parallel/I_\perp$. To rule out the possibility of depolarization of the fluorescent signal due to light scattering the membranes were labeled with DPH, their anisotropy was measured, and then the membrane preparations were diluted successively in PBS solution. This did not affect the anisotropy and proves that there is no depolarization due to light scattering. This was further proven by experiments where the ratio between the concentration of membranes (which cause the scattering) and DPH was varied. Substantial differences in fluorescent intensity were found but there was no effect on the anisotropy (see Results).

Calculation of Microviscosity. The data were plotted as the effect of temperature on the anisotropy (r) (which is the measurable parameter) and as Arrhenius plots describing the natural log of the microviscosity (in poise) as a function of $1/T$ (where T is the absolute temperature). The microviscosities were calculated by the Perrin equation which described the effect of viscosity on the rotational depolarization of a fluorophore as described by Cogan et al. (1973). Using the modified equation (Shinitzky and Barenholz, 1974)

$$r_0/r = 1 + [C_{(r)}(T\tau/\eta)] \quad (4)$$

where r and r_0 are the measured and limiting fluorescence anisotropies, respectively, T is the absolute temperature, τ is the excited state lifetime, η is the viscosity of the medium, $C_{(r)}$ is a parameter of the shape of the fluorophore and is dependent on the anisotropy (r). The calculations of the microviscosities were done using the calibration of DPH in purified White American Oil 35 as described elsewhere (Shinitzky and Barenholz, 1974). All the calculations, statistical correlations, and the plotting were done using a CDC 6400 computer.

Results and Discussion

Fluorescence Intensity and Fluorescence Polarization of the DPH in the VS Viral Membrane: Effect of the Ratio between DPH and VS Virus Protein. VS virus suspensions have a high turbidity per unit of lipid. This prompted us to study the effect of the DPH to VS virus ratio on the fluorescence polarization of DPH in order to find the optimal conditions to obtain reliable measurements of anisotropy and microviscosity values. This problem is probably present with most biological systems since viruses, cells, and most subcellular organelles have a high turbidity per unit of lipid. Other problems may be encountered with some biological membranes such as specific interaction between the probe used to monitor the membrane properties and one or more of the membrane components (Stubbs, G. W., Barenholz, Y., and Litman, B. J., in preparation). VS virus grown on BHK-21 cells was used for the preliminary studies since the virus yields are much superior to those of infected L cells. Pure VS virus (200 μg of protein) suspended in 4 ml of PBS was labeled at 39 °C with various amounts of DPH (0.25–5 nmol). This corresponds to ratios which range from 1 molecule of DPH per 300 molecules of lipid to 1 molecule of DPH per 16 molecules of lipid. This calculation is based on our finding that the mass ratio between the cholesterol plus total phospholipids to the total VS viral protein is 0.26 and the mole ratio between cholesterol and phospholipids is 0.72 (Moore, N. F., Barenholz, Y., and Wagner, R. R., manuscript in preparation).

Figure 1 presents data on fluorescence intensity and polarization of DPH in VS viral membrane which show the following: The total intensity (curves A and B) is linear with the DPH concentration only up to a ratio of 0.5 nmol of DPH per 200 μg of VS virus protein. Above a ratio of 1 nmol of DPH per 200 μg of VS virus protein, the total fluorescence intensity remains constant. These results were obtained at the two temperatures (39 °C, curve B, and 22 °C, curve A) indicated in Figure 1. However, $I_\parallel/I_\perp$ (curves D and E) appears to be constant for all DPH to VSV ratios.

To obtain better insight into the mechanism which prevents the linear dependency of the total fluorescence intensity with increasing DPH concentration, more virions were added (100 μg of protein) to all VS virus–DPH suspensions. Increase in fluorescence intensity was found at all ratios of DPH to VS virus above 0.5 nmol of DPH per 300 μg of total VS viral protein (Figure 1C). This increase occurred at the same rate as did the first insertion of probe and probably requires the transfer of probe molecules through the water to the added unlabeled VS virus. This mechanism is apparently different

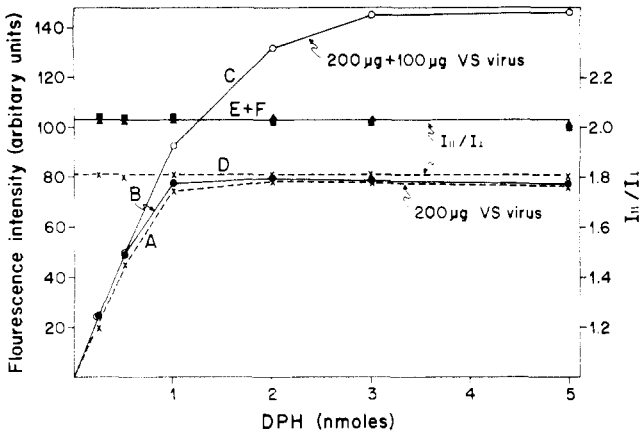

FIGURE 1: The effect of the ratio between DPH and VS virus on the total fluorescence intensity and ratio between $I_{\parallel}$ and $I_{\perp}$. Various amounts of DPH were interacted with VS virus (200 μg of protein) and measurements were performed when the penetration of the probe was complete as described in the Methods. The total fluorescence intensity F ($F = I_{\perp} + 2I_{\parallel}$) is indicated by the left ordinate and the right ordinate indicates the ratio of $I_{\parallel}$ to $I_{\perp}$. Curves A (X - - - X) and D (X - - - X) show the effect of concentration of DPH, with purified VS virus (200 μg of protein), on F and $I_{\parallel}/I_{\perp}$, respectively, at 22 °C. Curves B (● — ●) and E (■ — ■) show the same effects at 39 °C with the same samples. Curve C (○ — ○) shows the fluorescent intensity at 22 °C on further addition of VS virus (100 μg of protein) to the samples containing different concentrations of DPH. Curve F (▲ — ▲) shows the effect on the ratio of $I_{\parallel}$ to $I_{\perp}$ of the addition of an additional 100 μg of VS virus (protein).

from that noted by transfer of probe by collision between two populations of lipid vesicles (Lentz, B. R., Barenholz, Y., and Thompson, T. E., manuscript in preparation) or interactions between disks prepared from the outer rod segments and lipid vesicles (Stubbs, J. W., Barenholz, Y., and Litman, B. J., manuscript in preparation). In both of the latter cases, the transfer of the probe from one population to the other is much faster. It should be stressed that, although the addition of further VS virus strongly affects the total fluorescence intensity, there is no effect on the $I_{\parallel}/I_{\perp}$ ratio (Figure 1F). This rules out the possibility of probe–probe interactions such as energy transfer which will cause fluorescence depolarization (Weber, 1954).

These results might be explained by one of the following possibilities: (1) All the DPH is in the lipid bilayer of the membrane but there are deviations from the linear relationship between the concentration of the probe and the total fluorescence intensity at low optical density values. Although for all the samples used in the experiment the absorbance of the probe did not exceed 0.1 OD, the real concentration of the probe in the lipid (based on the fact that only the DPH present in the lipid bilayer will fluoresce) was possibly in the range of ca. 5-100 mM (based on lipid to protein ratio of 0.26 to 1 and a density of the lipid of ca. 1.0 g/ml). (2) The fluorescence intensity at any given temperature is proportional to the amount of DPH present in the hydrophobic region of the membrane. However, the amount of DPH present in the membrane is not proportional to the amount of DPH present in the virus suspensions and there is a limit to the amount of DPH that can penetrate into the bilayer per unit of membrane.

The effect of the ratio between DPH and the amount of membrane on the total fluorescence intensity and $I_{\parallel}/I_{\perp}$ ratio was also checked by using constant amounts of probe (2 nmol) for labeling with various amounts of virions (between 50 to 600 μg of protein per 4 ml of PBS, corresponding to 20–240 nmol of lipid). Because of strong light scattering we could not increase the amount of virus above 600 μg. At levels up to 160 nmol of lipid to 2 nmol of DPH the total fluorescence intensity was linear and therefore linearly proportional to the amount of virus. The slope of the curve is a tangent value of 1.0. Above 160 nmol of lipid per 2 nmol of DPH the slope becomes less dependent on the amount of membrane.

It should be noted that, when the mole ratio of lipids to probe was lower than 30, a slight drop of intensity (~8%) occurred when the temperature was reduced from 37 to 20 °C although the change in the $I_{\parallel}/I_{\perp}$ ratio with temperature was identical with that found for 1 nmol of DPH per 300 lipid molecules. This might be explained by having the limit of DPH to lipid molecules in the membrane (but not the environment of the DPH, or the interaction between DPH and its environment) affected by temperature.

These findings led us to the following conclusions:

(1) In order to obtain reliable data all experiments must be performed with a ratio of viral lipid molecules to DPH molecules greater than 200.

(2) There is an upper limit to the amount of probe that can be inserted in the hydrophobic region of the viral membrane at a level between 160 molecules of lipid to one DPH molecule and 80 molecules of lipid to one DPH molecule.

(3) Excess probe which did not fluoresce is intact and is not chemically modified.

(4) The excess of probe which did not fluoresce can be expressed by adding more virions, a process which requires transfer of probe to the added virus through the aqueous phase

Effect of Labeling with DPH on the VS Virus Properties. In this study DPH was used to monitor the dynamics of the hydrophobic region of the membrane of a biologically active system (i.e., VS virus) and it was important to demonstrate that the experimental conditions were not affecting the virus structure or infectivity. The effect of DPH-labeling on the virus properties was demonstrated by the following procedures:

(1) VS virus was incubated with DPH at 39 °C using the experimental conditions described above. Infectivity assays were performed on monolayer cultures of L-929 cells as previously described (Wagner et al., 1963) using an equivalent amount of unlabeled and labeled VS virus. No loss of infectivity occurred with the DPH-labeled virus.

(2) Electron microscope examination of negatively stained pure VS virus showed bullet-shaped particles with a high degree of membrane integrity. We were unable to differentiate between DPH-labeled and unlabeled virus preparations.

(3) Using radioactively labeled virus (^{14}C-labeled amino acid) we were unable to differentiate between DPH-labeled and unlabeled VS virus by equilibrium gradient analysis as described in the Materials and Methods. There was no evidence of formation of nucleocapsids or other virus debris on labeling with DPH.

(4) Labeling of VS virus at a range of temperatures, up to 39 °C, gave the same temperature-dependent anisotropy values. Furthermore the behavior was fully reversible as expected from the thermotropic behavior of a lipid phase. The only parameter which was affected by the temperature of labeling (up to 39 °C) was the rate of penetration of the probe as measured by t_{50} and t_{100} values.

Comparative Dynamic Properties of Intact Membranes and Liposomes of VS Virus and Host L Cells. Uptake of fluorescent probe and microviscosity were examined in membrane hydrophobic regions of VS virus grown in L cells, plasma membranes of uninfected L cells, and vesicles prepared from lipids extracted from VS virus and L-cell membranes. Growth and purification of VS virus and preparation of L-cell plasma membranes are described in Materials and Methods. Vesicles

TABLE I: Time Required for DPH to Reach 50% and 100% of Total Incorporation.[a]

System	t_{50} (min)	t_{100} (min)
VS virus grown in L-929 cells	1.2 ± 0.04	18.0
Liposomes prepared from extracted virus lipids	0.2 ± 0.04	3.0
Plasma membrane of L-929 cells (prepared by method of Caliguiri and Tamm, 1970)	0.7 ± 0.04	23.0
Liposomes prepared from extracted plasma membrane lipids	0.2 ± 0.04	4.5
Intact L cells	0.6 ± 0.04	13.0

[a] Results are calculated from the measurement of the increase of total fluorescence intensity as a function of time. Using equivalent ratios of DPH to lipid in plasma membrane, virus, and liposomes, the total fluorescence intensity was monitored until no further increase occurred. The time required for the total fluorescent intensity to reach half its maximum value (t_{50}) and the maximum value (t_{100}) for the various systems is shown.

TABLE II: Anisotropy of the Viral and Cellular L-929 Cell-Systems at Three Temperatures.

System	Anisotropy[a] 10 °C	25 °C	37 °C
VS virus	0.297	0.259	0.229
VS virus lipid vesicles	0.297	0.262	0.229
L cell plasma membrane (method of Perdue et al., 1971a,b)	0.273	0.218	0.189
L cell plasma membrane (method of Caliguiri and Tamm, 1970)	0.262	0.212	0.173
L cell plasma membrane lipids (method of Caliguiri and Tamm, 1970)	0.277	0.216	0.172
Intact L cells	0.253	0.208	0.178

[a] Anisotropy values were calculated from $r = (A - 1)/(A + 2)$, where r is the anisotropy and $A = I_{\parallel}/I_{\perp}$.

of the viral and cell membrane preparations were prepared from lipids completely extracted by the Folch method. Intact membranes and liposomes suspended in PBS were labeled with DPH as described above. All experiments were performed with virus or cell preparations in which the ratios of lipid molecules to DPH probe were 300 to 1.

In the first group of experiments, comparisons were made between the rate of DPH penetration into the hydrophobic region of the virus membrane, lipid vesicles made from the viral membrane, L-cell plasma membrane prepared by the method of Caliguiri and Tamm (1970) (method B), and vesicles made from the lipid extract of this plasma membrane. The results of this experiment are shown in Table I where the values of the time in minutes required to reach 50% (t_{50}) and 100% (t_{100}) of the maximal total fluorescence intensity are shown.

The general conclusions that can be drawn from these experiments are:

(1) In both the case of the virus and plasma membrane, the rate of DPH incorporation into the hydrophobic region of the lipid bilayer is much faster with the lipid vesicles than with the intact membrane. (In both cases the same amounts of lipids and DPH were used.) This differential uptake is probably attributable to the greater surface area available for probe penetration into the lipid vesicles than that of plasma membrane or virus. Two factors contribute to the relative surface areas of intact membranes and lipid vesicles: (a) the difference in size and (b) the presence of proteins in the surface. Removal of the viral spikes by proteases increases the rate of DPH penetration into the membrane of the otherwise intact VS virion (data not shown).

(2) The absolute values of total intensity per unit of lipid were almost identical for virions and lipid vesicles prepared from these virions as well as for the plasma membrane and the lipid vesicles prepared from the lipid extract of the plasma membrane. However, as demonstrated later the values for each pair of results differ due to differences in the fluorescence anisotropy.

(3) In all cases the fluorescence anisotropy of DPH was constant with time (Figure 1). The values obtained at 37 °C were ca. 0.229 for the virus grown on L cells and for the lipid vesicles prepared from these virions, respectively. Values of 0.173 and 0.172 were obtained for the plasma membrane fraction prepared by the method of Caliguiri and Tamm (1970) and for the lipid vesicles prepared from this membrane, respectively. The finding that total fluorescence intensities as well as the anisotropies in each membrane pair were very close indicates that in both cases there was no quenching of the DPH by the protein present in the intact membrane. The amount of protein present in the lipid vesicle preparation is less than 1% of the protein present originally in the viral membrane as determined by using cells that were growing on medium containing a ^{14}C-labeled mixture of amino acids.

The next set of experiments was designed to study the effect of temperature on the microviscosity (as calculated from the anisotropy) of the viral membrane, the lipid vesicles made from membrane, two preparations of plasma membrane prepared by different procedures, method A (Perdue et al., 1971a,b) and method B (Caliguiri and Tamm, 1970), and the lipid vesicles made from the lipid extracted from plasma membrane prepared by method B. The dependence of anisotropy on the temperature is summarized in Table II. It is very clear from these data that the viral membrane shows thermotropic behavior similar to that of lipid vesicles prepared from the virions. Similarly, the dependence of the anisotropy on temperature is very similar for the plasma membrane (method B) and the lipid vesicles prepared from this membrane. It is also very clear that there is a large difference in the absolute anisotropy values for all temperatures in the range of 5–40 °C with the viral membranes and the lipid vesicles prepared from this membrane giving higher values than those obtained for the L-cell plasma membrane and the lipid vesicles made from the plasma membrane.

Since the theoretical relationships between the anisotropy and the temperature are complicated, microviscosities (poise) were calculated as described in Methods. The dependence of the microviscosity (η) on the absolute temperature followed the exponential relationship, $\eta = Ae^{\Delta E/RT}$, or the linear relationship, $\ln \eta = \ln A + (\Delta E/RT)$ (Arrhenius plot). In these equations, η is the apparent microviscosity, A is a constant characteristic of the system, ΔE is the flow activation energy, R is the gas constant, and T is the absolute temperature. The natural logarithm of the microviscosity is then simply a linear function of $1/T$ for a homogeneous phase.

The data are plotted (Figure 2) as Arrhenius plots describing $\ln \eta$ as a function of $1/T$. From these curves the following parameters can be determined.

(1) Phase transition or phase separation. In the case of a phase transition a break in the linear curve and a drastic change of the slope occur which can be easily observed (Shinitzky and Barenholz, 1974). Transitions obtained using DPH correspond

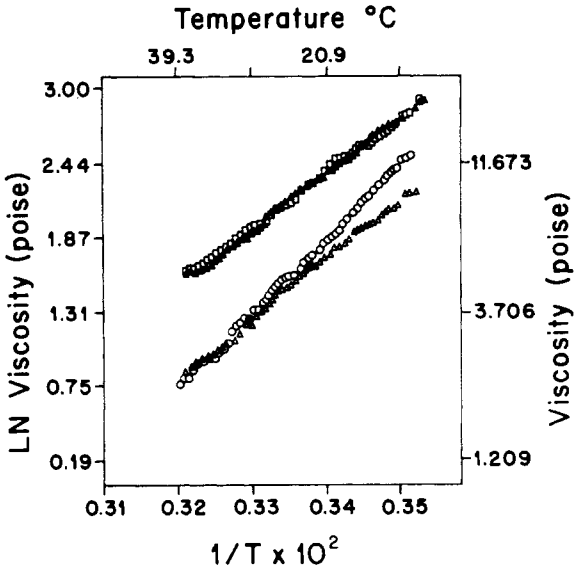

FIGURE 2: Dependence of the natural logarithm of viscosity (in poise) on temperature for intact VS virus (□), liposomes made with the extracted virus lipids (▲), plasma membrane of L-929 cells prepared by Method B (Caliguiri and Tamm, 1970) (△), and liposomes prepared from the plasma membrane (Method B) lipids (○). Reference points showing actual viscosity (in poise) and actual temperature are indicated on the right ordinate and top abscissa, respectively.

closely with transitions obtained by NMR, calorimetric, and ESR studies (Barenholz, Y., and Thompson, T. E., manuscript in preparation; Lentz, B. R., Barenholz, Y., and Thompson, T. E., manuscript in preparation; Shimshick and McConnell, 1973). A phase transition will cause a large drop in the correlation coefficient of linearity (R) as well as an increase in the standard deviations of A (calculated from the intercept) and of the slope. Phase separations can also be observed, but only if the amount of lipid involved is large enough to show deviation from the linear curve, since this measurement can give only average properties of the membrane.

(2) The flow energy of activation (ΔE) for the probe can be calculated since the slope of the curve is described by $\Delta E/R$.

(3) A can be calculated from the intercepts of the Arrhenius plots.

(4) In the case of linear curves (or for linear parts of the curve), the similarity between different curves can be shown by comparing the slope and the intercept of the Arrhenius plots. The values calculated from the curves, including the standard deviation of the slope and the intercept, are shown in Table III. As shown in Table III all the curves plotted in Figure 2 have no distinct thermotropic phase transition. All the curves have a correlation coefficient of linearity above 0.9970 and the standard deviation of the intercept and the slope is in the range of ±1%. For example, a comparable experiment with multibilayered liposome made of dioleoyllecithin will give similar parameters for the Arrhenius plot (Lentz, Barenholz, and Thompson, in preparation); however, for multibilayered liposomes made of dipalmitoyllecithin, which undergo a thermotropic transition at 41.3 °C, the correlation coefficient of linearity will be much lower, ca. 0.8 or less, and the standard deviations for the intercept and the slope are larger than ±10% (Lentz, B. R., and Barenholz, Y., unpublished data). The curve of dipalmitoyllecithin has a break in the slope at ca. 41.0 °C. All the curves in Figure 2 show some deviations from a straight line which might be explained by phase separation of relatively small numbers of lipid molecules. Fluorescence polarization methods, as with NMR and ESR methods in use today, describe only average properties of the membrane. Much more sophisticated techniques have to be developed in order to trace phase separation involving small numbers of lipid molecules, such as very sensitive calorimetric methods or labeling of the lipid molecules that might be involved in the phase separation using either deuterium or ^{13}C for NMR studies. In summary, the phase separation involving small numbers of lipid molecules cannot be detected by using the fluorescence polarization technique.

It is obvious that the plasma membranes and the lipids derived from the plasma membranes have much lower microviscosity values than the virus and virus lipids throughout the temperature range.

The reproducibility of the fluorescence anisotropy and therefore microviscosity values were better than 2% for measurements that were done on the membranes or lipid vesicle preparations derived from the same cultured cells or the same virus preparation. However, some differences were found for measurements that were done with the same membranes derived from different cells or virus preparations. But even when such differences were found, the relative difference between the microviscosity or anisotropy remained unaffected for the virus membrane to plasma membrane.

Table III demonstrates the maximal differences that were obtained for plasma membrane from different preparations. Experiment 1 shows the highest microviscosity values and experiment 2 the lowest values. Experiment 2 also demonstrates the usual differences obtained between plasma membrane fractions prepared by the two different methods. However, it must be stressed that the extent of the differences is dependent on the procedure for plasma membrane preparation since the plasma membranes prepared by different methods are not identical. Preparation A (Perdue et al., 1971a,b) is more viscous than preparation B (Caliguiri and Tamm, 1970). This finding is supported by the data obtained by McSharry and Wagner (1971) showing that plasma membrane prepared from the same cell line by two methods had clearly different lipid composition. Such differences might account for the differences in microviscosity. From this the question arises as to which of the plasma membrane preparations can be used for comparison with the viral membrane?

The close correspondence between the viscosity of the intact virus membrane and the lipid vesicles derived from it indicates that for both systems the DPH is probing a very similar environment. The DPH is evenly distributed throughout all regions of the hydrophobic areas of the lipid bilayer without any preference for rigid or fluid regions as shown for lipid vesicles by Lentz, B. R., Barenholz, Y., and Thompson, T. E. (manuscript in preparation), or for systems containing disks made of the rod outer segment (Stubbs, G. W., Barenholz, Y., and Litman, B. J., manuscript in preparation). The value calculated for membrane viscosity represents the average microviscosity of the entire lipid bilayer. This will rule out the possibility that either the VS viral glycoprotein or matrix protein, or both proteins, are the main factors causing the high viscosity of the viral bilayer. A similar observation was obtained by Stoffel and Bister (1975) for VS virus grown on BHK-21 cells using ^{13}C NMR.

Both the plasma membrane of the L cells and the lipid vesicles prepared from a total lipid extract have very similar viscosities which are lower than those of the viral membrane. This again tends to rule out the effect of protein on the average microviscosity. Therefore the primary reason for the differences in microviscosity between the plasma membrane and the viral membrane would appear to be due to differences in lipid

TABLE III: Microviscosity in Poise at Three Temperatures and Comparative Data Calculated from the Arrhenius Plots.

System	Microviscosity (P) 10 °C	25 °C	37 °C	ΔE (kcal/mol)	Intercept (P × 10^5)	Correlation Coeff
Experiment 1						
VS virus	14.93	7.60	4.92	7.46 ± 0.06	−10.6 ± 0.09	0.9987
VS virus lipids	14.92	7.73	4.90	7.68 ± 0.06	−10.99 ± 0.1	0.9983
L cell plasma membrane (method of Caliguiri and Tamm, 1970)	8.05	4.00	2.29	8.25 ± 0.07	−12.63 ± 0.12	0.9980
L cell plasma membrane lipids (method of Caliguiri and Tamm, 1970)	10.1	4.20	2.22	10.1 ± 0.06	−15.66 ± 0.1	0.9989
L cells	7.12	3.90	2.55	6.89 ± 0.008	10.32 ± 0.13	0.9982
Experiment 2						
L cells plasma membrane (method of Perdue et al., 1971a,b) (method A)	9.60	4.30	2.78	8.07 ± 0.08	−12.15 ± 0.13	0.9977
L cells plasma membrane (method of Caliguiri and Tamm, 1970) (method B)	6.80	3.25	1.68	9.22 ± 0.08	−12.63 ± 0.1	0.9989

composition. Similar results were obtained by Robinson et al. (1972) showing that the sarcoplasmic reticulum membrane has similar relaxation times to liposomes prepared from their lipid extract.

The main factor in lipid composition which is well established as a determinant of membrane microviscosity is the ratio between cholesterol to total phospholipids (Hubbell and McConnell, 1971; Chapman and Pemkett, 1966; Cogan et al., 1973; Oldfield and Chapman, 1971; Inbar and Shinitzky, 1974). Another factor is the degree of unsaturation of the phospholipid and sphingolipid acyl chains (Lentz et al., manuscript in preparation; Chapman and Wallach, 1968). We would like to stress that by no means are these the only parameters which will affect membrane microviscosity, which is probably determined by a very complicated average of the interactions among all lipid species present in any membrane.

As shown by McSharry and Wagner (1971) there are drastic differences in the lipid composition of the plasma membrane prepared from L cells and the membrane of VS virions grown on L cells. We should stress that the main difference is not in the ratio of cholesterol to total phospholipids, which is very similar in the plasma membranes and the VSV membranes. However, big differences in phospholipid composition exist in the two membranes. This is demonstrated by calculating the mole ratio between the various phospholipids. Comparing plasma membrane prepared by the FMA[1] method (McSharry and Wagner, 1971) with the viral membrane, one can see that the virus contains higher amounts of SPM,[1] PE,[1] and PS[1] than the plasma membranes. When these data are expressed as ratios it is apparent that in the viral membrane the SPM to PC ratio is four times higher. The PE to PC ratio and the PS to PC ratio are both about five times higher, in the virus membrane than these ratios in the FMA plasma membranes.

According to Stoffel and Bister (1975) the high rigidity of the VS viral membrane is due to the high cholesterol to total phospholipid ratio. To us it seems that other factors are involved as well since the plasma membrane of L cells and the VS virus membrane have similar cholesterol to total phospholipids ratio (McSharry and Wagner, 1971) but its microviscosity is lower. We assume that the other possible factors that contribute to the higher microviscosity are the higher ratio of SPM to PC and to some extent also the higher degree of saturation of the viral membrane acyl chains (McSharry and Wagner, 1971). We cannot rule out effects of PE/PC ratios and PS/PC ratios on the membrane microviscosities especially since this ratio is much higher in the virus than in the plasma membrane. Data on lipid composition of various viruses with comparison with the plasma membrane of their host cells (Lenard and Compans, 1974) show that in most of the systems similar differences between the viral membranes and the plasma membranes exist. It is important to correlate these differences with the microviscosity of the viral envelope and their host cell plasma membrane. Preliminary data obtained by us indicates that in many cases the microviscosity of the viral membrane is higher than that of the plasma membrane of the host cells. These findings indicate at least some specificity in the way in which an enveloped virus selects its membrane lipids from its host cell.

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Evidence for Phase Boundary Lipid. Permeability of Tempo-choline into Dimyristoylphosphatidylcholine Vesicles at the Phase Transition†

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ABSTRACT: The existence of distinct regions of mismatch in molecular packing at the interfaces of the fluid and ordered domains during the phase transition of dimyristoylphosphatidylcholine vesicles has been demonstrated by measuring the temperature dependence of the permeability to a spin-label cation and comparing this with a statistical mechanical calculation of the fraction of interfacial lipid. The kinetics of uptake and release of the 2,2,6,6-tetramethylpiperidinyl-1-oxycholine (Tempo-choline) spin label by single-bilayer dimyristoylphosphatidylcholine vesicles were measured using electron spin resonance spectroscopy to quantitate the amount of spin label present within the vesicles after removal of the external spin-label by ascorbate at 0 °C. Both the uptake and release experiments show that the Tempo-choline permeability peaks to a sharp maximum at the lipid-phase transition, the vesicles being almost impermeable to Tempo-choline below the transition and having a much reduced permeability above. The temperature profile of the permeability is in reasonable quantitative agreement with calculations of the fraction of interfacial boundary lipid from the Zimm and Bragg theory of cooperative transitions, which use independent spin-label measurements of the degree of transition to determine the cooperativity parameter. The relatively high intrinsic permeability of the interfacial regions ($P \sim 0.2\text{–}1.0 \times 10^{-8}$ cm/s) is attributed to the mismatch in molecular packing of the lipid molecules at the ordered-fluid boundaries, which could have important implications not only for permeability in natural membranes (e.g., in transmitter release), but also for the function of membrane-bound enzymes and transport proteins.

The possible functional importance of the lipid-phase transition and of the conditions of lateral lipid-phase separation is now well established, as a result of many studies on both biological and model membranes (see e.g., Marsh, 1975).

The increase in fluidity at the phase transition has been shown to enhance the activity of enzymes and specific transport systems within the membrane (Overath and Träuble, 1973; Esfahani et al., 1972) and the possible physiological significance of lipid-phase transitions in triggering these membrane functions has been emphasized (Träuble, 1972; Träuble and Eibl, 1973). Lateral phase separation of lipids within *Escherichia coli* plasma membranes has also been shown to have a dramatic effect on sugar transport by the membrane (Linden et al., 1973).

These effects can be attributed to the more expanded and more flexible nature of the lipid chains in the fluid state relative to the ordered state of the chains below the phase transition (Hubbell and McConnell, 1971; Marsh, 1974a). Conformational changes of embedded proteins are thus facilitated in the fluid state above the phase transition, and transitions from the expanded to the condensed state give rise to a high lateral compressibility of membranes in a state of lateral phase separation. This in turn also facilitates conformational transitions of membrane proteins and could also accommodate the introduction of newly synthesized proteins and lipids into the membrane (Linden et al., 1973).

Of possibly equal importance are the properties of the

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