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Probing the Critical Unilamellar State of Membranes

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Abstract. Aqueous dispersions of membrane phospholipids comprised of multilamellar vesicles (MLVs) will spontaneously transform to a stable unilamellar structure equivalent to the membrane bilayer, but only at a critical temperature T*. Since much of the evidence for this transformation derives from equilibrium thermodynamic studies, a description of the molecular and topological events occurring as the critical unilamellar state assembles has not previously been possible. Here we report experiments that provide evidence of a spontaneous topological change from MLVs towards unilamellar vesicles (ULVs) at T*. By applying a shearing stress to vesicle suspensions we have observed a decrease of approximately 25% in the force required to cause bilayers to leak; this decrease is confined to temperatures near T*. The T* values observed agree with those previously obtained by equilibrium methods. Using the method with total lipid extracts from normal biological membranes confirms that T* equals the physiological temperature of the original membranes.

Key words: Critical bilayer state — Unilamellar — Vesicles — Membrane assembly — Phospholipid bilayers

Introduction

Phospholipids in aqueous dispersions will spontaneously assemble as a stable unilamellar structure equivalent to the membrane bilayer, but only at a critical temperature T*. At this critical point multilamellar vesicles (MLVs) transform to a new equilibrium state consisting only of unilamellar vesicles (ULVs) (Gershfeld, 1989*a,b*); for all other temperatures above and below T* only MLVs form. The evidence for the transformation to the critical unilamellar state is largely based on the thermodynamic properties of phospholipid dispersions as monitored by the equilibrium behavior of the lipid films which form at the contiguous air-water surface, (Gershfeld & Tajima, 1979; Tajima & Gershfeld, 1985; Gershfeld, 1989*b*). These studies show that at T* an unencumbered bilayer forms spontaneously and rapidly, completely covering the air-water surface; this bilayer exhibits both the density (Tajima & Gershfeld, 1985) and permeability (Ginsberg & Gershfeld, 1985) of a typical unilamellar structure. The conditions for the formation of this unilamellar structure are completely defined by the critical temperature T* and the lipid composition (Gershfeld, 1989*a,b*).

These conditions of temperature and composition which define the critical unilamellar state are general and are applicable to all phospholipid dispersions which form MLVs including the total lipid mixtures obtained from cell membranes. For the complex membrane lipid mixtures from a wide variety of organisms and tissues we have found that T* equals the physiological temperature Tp of the cell from which the lipids were extracted (Gershfeld, 1986; Ginsberg, Gilbert & Gershfeld, 1991). Since the critical unilamellar state forms in the dispersions under the identical conditions of temperature (T* = Tp) and composition that exist in the cell, the necessary and sufficient conditions to define the state, this result can only mean that the unilamellar structure of the cell membrane and of the dispersion are identical, and that Tp is a critical point.[1]

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[1] Bilayers which form in dispersions have the lipids distributed symmetrically across the bilayer, while in virtually all cell membranes they are asymmetrically distributed. We have deduced that because T* = Tp the membrane bilayer and the bilayer of the dispersion are neces-

In the present study the response of liposomes to a shearing force is examined to further characterize the critical bilayer state. We have chosen this technique because the shear modulus is a property reflecting the intermolecular forces which maintain bilayer structure, and therefore allows us to monitor the molecular properties of the critical state as the MLVs are transformed into ULVs. Since this transformation necessarily involves opening of bilayers to allow release of internal lamellae, such openings, limited to temperatures near T*, would lead to a weakening of vesicle structure. Bilayer weakening on exposure to varying shear stress is quantified as the bilayer rupture threshold, τ_r the applied shear at which bilayers become ''leaky''. The release of a marker from intravesicular aqueous spaces is a sensitive indicator of bilayer rupture (Ginsberg, 1978). For this study we used a fluorescent probe, initially trapped within MLVs at self-quenching concentrations (Weinstein, et al. 1977; Chen & Knutson, 1988). When MLVs rupture, dye in the interlamellar aqueous spaces enters the external solution, becomes diluted and fluoresces.

The bilayer rupture threshold was measured for liposome suspensions of dimyristoylphosphatidylcholine (DMPC) and egg lecithin (EggPC), materials with T* values previously determined by equilibrium methods. We show that bilayer weakening occurs, but that it is restricted to temperatures close to T*. An analysis of bilayer weakening in MLVs as a characteristic property of the MLV-ULV transformation is presented, and we show that the process directly mirrors the formation of the unilamellar state in the air-water surface at T*. The shear stress technique is applied to the lipids of various membranes, where it is again demonstrated that T* = Tp.

Materials and Methods

Suspensions of MLVs were prepared from DMPC, EggPC (both from Avanti Polar Lipids, Alabaster, AL; purity >99%, used without further purification) and total lipid extracts of biological membranes (human and rabbit erythrocytes, rabbit and rat central nervous system), obtained as described previously (Gershfeld, 1986; Ginsberg et al., 1991). The vesicles were loaded with the fluorescent dye 5-(and 6-) carboxyfluorescein (obtained as mixed isomers from Molecular Probes, Eugene, OR) by suspending dry lipid in a 0.1 M solution of the dye as sodium salt (pH 7.3) and vortexing vigorously. For most natural lipid mixtures this was sufficient to form a visually uniform suspension. With DMPC, the suspensions contained many large particles which were further dispersed by several cycles of heating the suspension to 50°C, vortexing, and then cooling to 0°C. The extravesicular dye solution was then removed by passing the suspensions through a Sephadex G-25 column; typically ~50 μg of lipid was suspended in 2 ml of a 0.135 M NaCl solution at pH 7.3, buffered with 10 mM HEPES.

Fluorescence was measured with a SLM 500C spectrofluorimeter (SLM Instruments), excitation wavelength 492 nm, the excimer emission was monitored at 520 nm. A dilution factor of approximately 10^5 on release of all the trapped dye was estimated. This instrument can readily measure 1% of this concentration. Thus, very small leaks due to the applied shear could be detected. The total amount of trapped dye was measured by adding a solution of a nonionic surfactant to the dispersion to release all remaining trapped dye at the end of an experiment. In no case did the amount of dye leakage exceed 50% of the amount initially present. All fluorescence measurements were controlled by computer software developed for specific timed sequences. Constant temperature control (±0.2°C) was maintained by Peltier modules attached to the cuvette holder of the instrument. All procedures were routinely performed under nitrogen to minimize decomposition by oxygen of lipids from natural sources.

Measurement of the Bilayer Rupture Threshold, τ_r

Application of a shearing force to vesicles with simultaneous monitoring of fluorescence was achieved by suspending a rotating disk in a lipid dispersion within a cuvette (*see* Fig. 1*a*). In general, the shear stress τ generated by a rotating disk in a fluid (Dorfman, 1963) depends on the viscosity of the fluid η, disk radius R, and angular velocity ω as

$$\tau = \eta R\omega/s \quad (1)$$

where s is the minimum spacing between the edge of the disk and the wall of the cuvette. Preliminary studies with DMPC dispersions using a nominal spacing for s of .05 cm indicated that bilayer rupture could not be achieved unless disk speeds exceeded 10^5 RPM. Such shear stresses could be attained, but they were accompanied by an unacceptable increase in temperature of about 5–10°C. We chose instead to increase the viscosity of the medium, thereby lowering ω, by introducing small glass beads of known dimensions into the dispersion.

Beads disturb the laminar flow of fluids and create small eddy currents whose scale of motion is of the order of the bead radius r. An effective eddy viscosity develops that is greater than the viscosity of the dispersing fluid (Levich, 1962). From Eq. (1) the increase in viscosity will reduce the angular velocity necessary to produce a given shear. The eddy viscosity, $\eta_e \sim R\omega r$, (Levich, 1962) when substituted into Eq. (1) gives the dependence of shear stress on bead size as:

$$\tau \sim r\omega^2 \quad (2)$$

This relation was tested using glass beads and measuring τ_r for DMPC vesicles. With 215 and 115 μm diameter glass beads bilayer rupture occurred at values of ω equal to 1.21×10^3 and 1.67×10^3 rad/sec, respectively, for the same dispersion of DMPC. From equation (2) $r\omega^2$ is constant, and therefore τ_r is independent of the hydrodynamic conditions of the experiment. Additional evidence that the intrinsic me-

sarily in the same state. This suggests that membrane assembly may occur by an equilibrium process that initially yields a bilayer with lipids symmetrically distributed but metabolic processes are present which redistribute the lipids across the bilayer without any overall compositional modifications. It is of interest to note therefore that in red blood cells (Seigneuret & Devaux, 1984; Zachowski et al., 1986) and platelet plasma membranes (Suné et al., 1987) it has been demonstrated that the asymmetric lipid distribution is maintained by metabolism; a slow relaxation of the lipid composition towards a symmetrical, equilibrium distribution across the membrane has been noted when metabolism is disengaged (Seigneuret & Devaux, 1984; Williamson et al., 1992; Wilson, Richter-Lowney & Daleke, 1993).

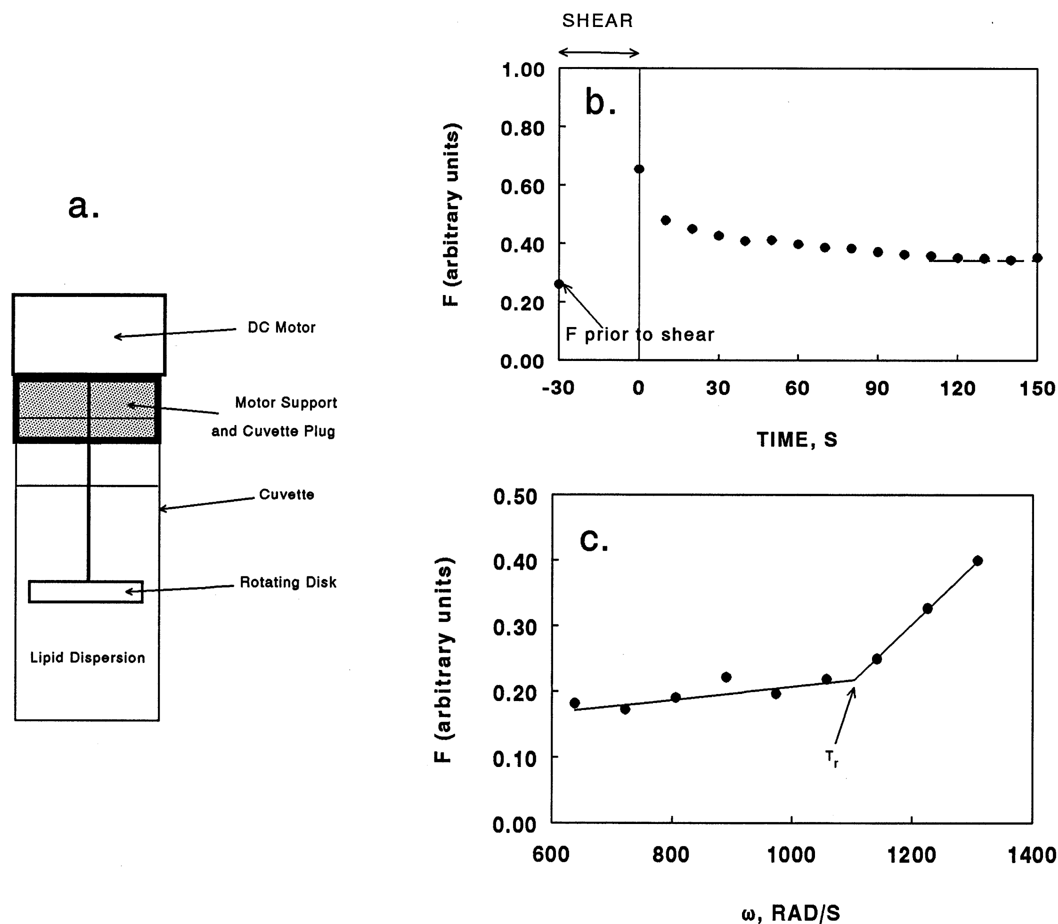

Fig. 1. (*a*) Experimental chamber for monitoring the influence of shearing force on lipid dispersions. A stainless steel rotating disk was suspended in the middle of a disposable cuvette from a small DC motor (MicroMo Electronics). All fluorescence measurements were performed in an atmosphere of nitrogen, the housing support of the stirring motor being constructed to fit snugly into the top of the cuvette thereby providing a gastight seal. To avoid scattering of the incident spectrofluorimeter beam by the disk and the supporting shaft, the cuvette was masked so that only the dispersion below the disk was illuminated. The disk radius was 0.45 cm, chosen to fit cuvettes with a cross-sectional area of 1 cm^2. (*b*) Fluorescence readings F for a typical vesicle suspension (EggPC) plus 215 μm glass beads (Applied Science Laboratory) (*see text*) after stirring at a selected disk speed for 30 sec. Fluorescence intensity was monitored every 15 sec for 15 readings in total. It decreased rapidly with time for the first five readings due to settling of the beads, followed by a more gradual decline primarily due to the rising of small gas bubbles which form due to rotation of the disk. The last five readings were averaged to give a value which reflected dye that had leaked from vesicles into the solution due to the applied shear stress, plus a minor contribution from partially quenched dye in the interlamellar spaces of vesicles. We have confirmed that dye leakage has occurred by centrifuging the shear stressed dispersion and measuring the fluorescence of the supernatant. (*c*) Measurement of τ_r for dye leakage from vesicles. The experiment in Fig. 1*b* was repeated at varying disk velocity ω and final fluorescence intensities *F* plotted as a function of ω. Little change in dye leakage was observed until the disk speed reached a value where the rupture threshold was exceeded, whereupon increasing speeds yielded increasing leakage of dye. The bilayer rupture threshold τ_r was characteristic of each lipid dispersion and temperature; τ_r measurements were reproducible to ±3%.

chanical properties of the vesicles are unaffected by the presence of the beads is provided in Fig. 2*b* (*see below*). The majority of the studies were performed with 215 μm beads because the angular velocities required to exceed τ_r were then in the most reproducible region of the DC motor driving the disk, and were low enough that the temperature near the disk rose less than 0.2°C. In general, we apply the shearing force for intermittent periods of 30 sec to minimize any temperature rise.

By varying ω, the relative influence of shear stress on a lipid dispersion could be examined. The bilayer rupture threshold τ_r was obtained by increasing the disk speed until the minimum velocity to produce dye leakage from vesicles was reached (Fig. 1*b* and *c*).

Results

Figure 2*a* and *b* shows the influence of temperature on τ_r for EggPC and DMPC, respectively. For both lipids the bilayer rupture threshold τ_r decreases over a narrow tem-

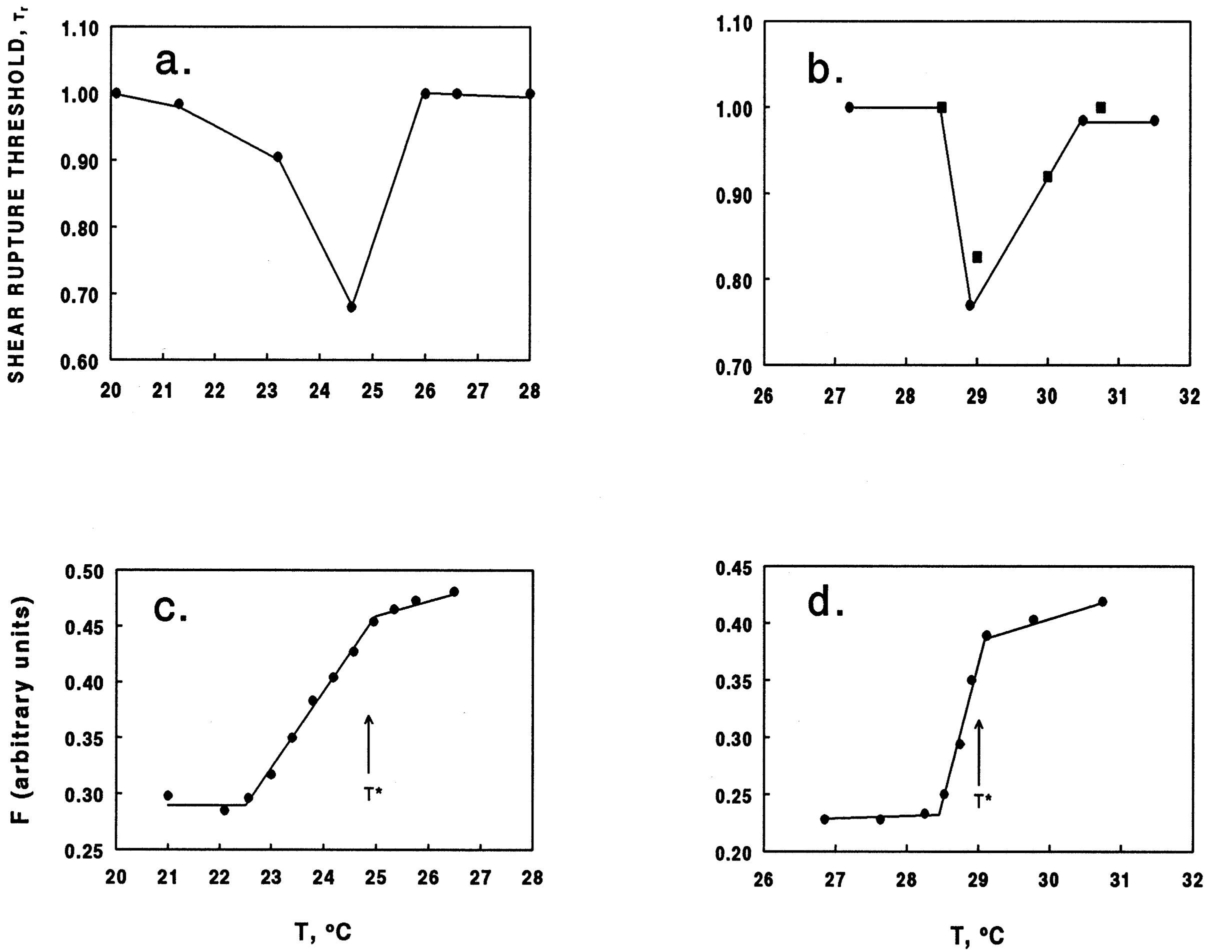

Fig. 2. Influence of temperature on the mechanical properties of EggPC and DMPC vesicles. (*a* and *b*) Shear rupture threshold τ_r for EggPC and DMPC vesicles, respectively; glass beads size: (●) 215 μm, (■) 115 μm. (*c* and *d*) Temperature scanning procedure for measuring T* of EggPC and DMPC, respectively. Fluorescence intensity *F vs.* temperature, *see text for details.*

perature interval beginning at 1–3°C below T*. At T*, a minimum τ_r is reached, approximately 25% lower than that outside this narrow temperature interval. The decrease in τ_r at T* is similar for DMPC and EggPC probably because at T* all bilayers are in the liquid crystalline state, (Gershfeld, 1989*a,b*) with similar mechanical properties. Values of T* for these lipids have previously been determined by equilibrium methods—DMPC: 29°C (Tajima & Gershfeld, 1985; Gershfeld et al., 1993); EggPC: 25°C, (*unpublished data*). Data for DMPC were obtained using 215 and 115 μm beads. The same relationship between τ_r and T* was observed, indicating that these properties are characteristic of the lipid and independent of the hydrodynamic conditions of the experiment.

Thus, for both EggPC and DMPC bilayer weakening, as evidenced by a fall in rupture threshold, is restricted to the temperature interval of the MLV-ULV transformation predicted from equilibrium thermodynamic studies (Gershfeld & Tajima, 1979; Tajima & Gershfeld, 1985; Gershfeld; 1989*b;* Gershfeld et al., 1993). The minimum τ_r is reached at T*, where the transformation to the unilamellar state is completed. When temperatures exceed T*, the bilayer rupture threshold characteristic of MLVs is recovered.

The unique mechanical properties of bilayers at T* were further demonstrated by measurements of dye leakage upon extended application of a constant shear stress at temperatures in the region near T* (Fig. 3). For all temperatures apart from T* itself, leakage of dye reaches a limit independent of the time the shearing force is applied. Thus, at T = 28.3° and 28.8° immediately below T*, and 29.4°C, just above T*, fluorescence increased 3–5% with 1 min of the applied shear stress, and remained unchanged with further application of the constant shear stress. However, at T* dye leakage continues without approaching any apparent limit on each application of the shear stress. For temperatures outside the critical region this applied shear stress did not affect dye leakage.

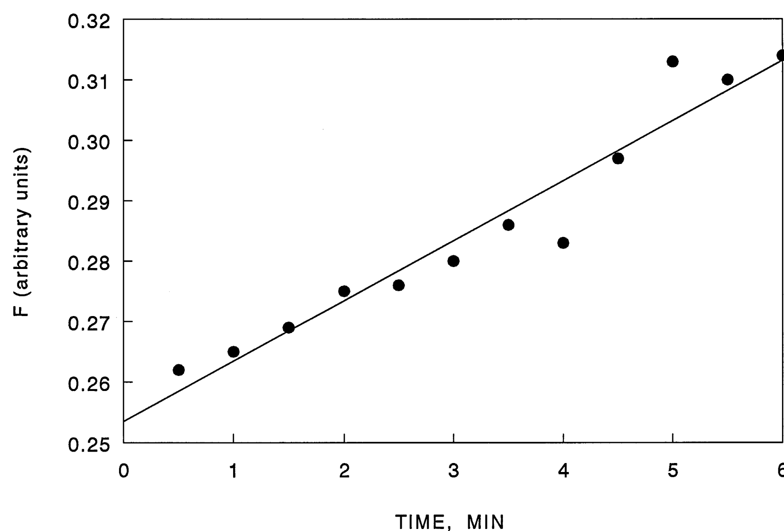

Fig. 3. Effect of continuous application of shear stress on fluorescence intensity F of a DMPC dispersion in the critical region near T* = 29°C. A constant shearing force, slightly less than the bilayer rupture threshold at a temperature outside the critical temperature range, was applied for repeated discrete periods of 30 sec (to a total of 5–6 min). At T* leakage continued upon each application of the shear stress; the solid line was calculated by linear regression, $r = 0.967$, For T = 28.3° and 28.8°C, immediately below T*, and 29.4°C, just above T*, (*data not shown*) a small (~3–5%) increase in fluorescence was observed for the first minute of applied shear. After this initial period of applied stress dye leakage reached a limit, within the experimental error of our measurement, and remained constant independent of the time stress was applied. At temperatures below and above the critical region the applied shear stress had insignificant effect on dye leakage (*see* Fig. 2*d*).

As values of T* previously determined by equilibrium methods for DMPC and for EggPC agree with the present studies, the mechanical approach was adapted to provide a rapid temperature scanning procedure for measuring T* (Fig. 2*c* and *d*). At a temperature where MLVs normally form, i.e., either below or above T* for the lipid in question, a constant value for the applied shear was chosen that was just below τ_r for the dispersion. The temperature was scanned over the entire interval encompassing T*. By applying bursts of this shear force at each temperature, T* was identified, as dye leakage only occurred at temperatures near it. A stress applied for 1 min (two 30-sec bursts) was sufficient to indicate whether vesicles were leaky at each temperature (*see also* Fig. 3). For both DMPC and EggPC leak is detected at the lowest temperature where bilayers begin to weaken due to the process of transformation to ULVs. With increasing temperature, vesicle leakage induced by shear stress continues until T* is reached. At temperatures exceeding T* dye leakage is diminished significantly because the processes that lead to formation of ULVs are now absent. In the absence of applied shear only relatively trivial amounts of dye leakage may be observed (1–2%/hr), in agreement with other studies in which this dye was used to monitor bilayer leakage (Weinstein et al., 1977).

To demonstrate that the shear stress method for determining T* is equally applicable to the more complex lipid mixtures in cell membranes, dispersions of the total lipid extracts from five different tissues were prepared. All the samples gave similar results, and the complete temperature scans for two of them are presented in Fig. 4. In each case the temperature range for the transformation from MLVs to ULVs is about 3–5 degrees. This is somewhat larger than the corresponding range observed with DMPC and EggPC (Fig. 2*c* and *d*), and probably reflects the wider distribution of lipid components. However, the general characteristics of the phenomenon are essentially the same in all cases, with a sharply defined transformation exhibited at T*. The values of T* for all five biological membranes obtained by this method are presented in the Table and compared with the physiological temperatures Tp of the source tissues.

Discussion

Since T* is unaffected by the shearing force, the molecular processes underlying the formation of the critical unilamellar state are also unaffected; the applied shear simply accelerates the processes that normally occur when MLVs are transformed to ULVs. What are these processes? Within the narrow temperature interval near T*, where bilayer weakening occurs, each 30-sec burst of shear can release approximately 5% of the total amount of dye in the interlamellar space (Fig. 2*c* and *d*). Dye loss to this extent is consistent with our initial premise that the MLV-ULV transformation must involve opening of bilayers to permit release of internal lamellae. As only a limited amount of dye is released at each of these temperatures below T*, bilayer opening may be regarded as a stochastic process in which only a certain population of lipid molecules has sufficient energy to form these openings. As T* is approached, this population of molecules increases. At T*, where dye loss from MLVs appears to continue unabated (Fig. 3), the entire population of molecules can participate in the transformation to ULVs, and bilayer opening will continue as long as the shear stress is applied or until completion of the transformation. When T > T* the equilibrium state is once more the MLV, and the bilayer opening process is absent.

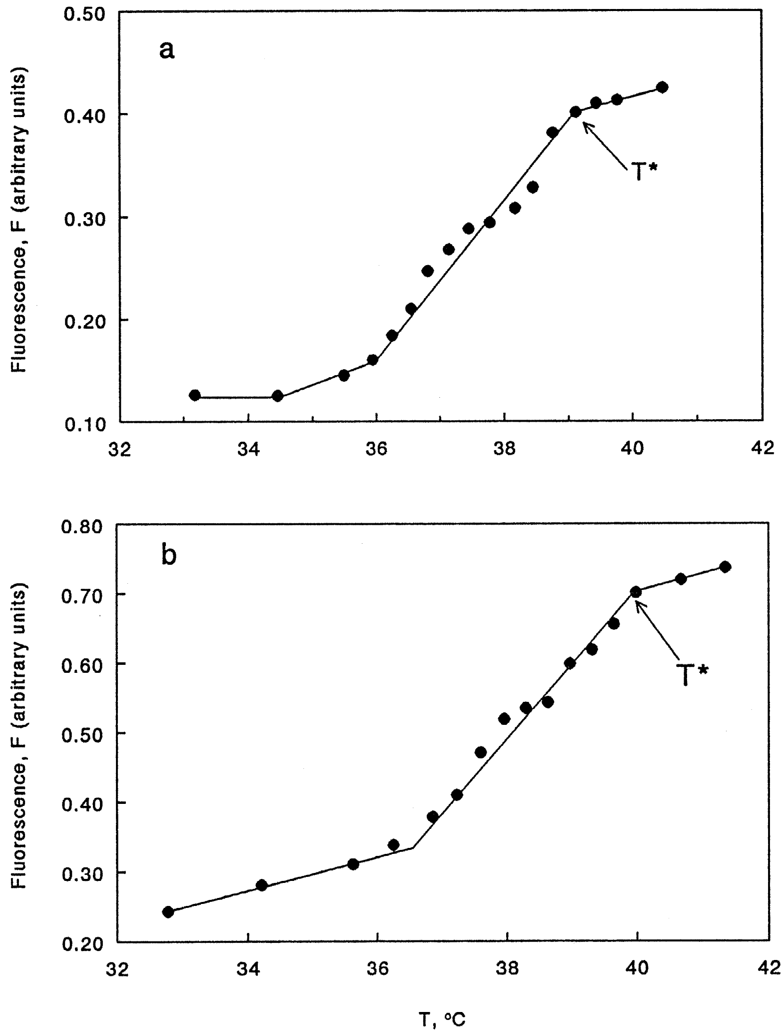

Fig. 4. Scanning method for measuring T* of dispersions containing the total lipid extracts of: (*a*) rabbit red blood cells (*b*) rat cerebrum.

Support for these conclusions is provided by recent calorimetric studies. According to the heat capacity (Cp)-temperature phase diagram for DMPC, a continuum of MLV states exists in a narrow temperature interval approaching T*; once T* is reached only ULVs are present (Gershfeld et al., 1993). This anomaly in Cp parallels the narrow temperature interval of bilayer weakening as indicated by the decrease in τ_r. The finite limit to dye loss at temperatures just below T* is to be expected within this framework i.e., if the bilayer is in a unique state at each of these temperatures. Only at T* does dye release continue apparently ceaselessly, the transformation from MLVs proceeding until all bilayers are unilamellar.

Bilayer weakening offers a possible explanation for how a bilayer might form in the air/water surface at T*. For poorly soluble phospholipid dispersions transport of lipid to the air/water surface is primarily from liposomes which enter the interface by Brownian motion. If a portion of a liposome particle in the interface is exposed to air, the outermost bilayer of this portion of the liposome will be subjected to a Laplace pressure that is a function of the curvature and surface tension at each of its surfaces: hydrated bilayer/air for the external and hydrated bilayer/water for the internal surface. The surface tension γ for the exterior is ~70 dynes/cm, and for the interior surface $\gamma \approx 0$ (Gruen & Wolfe, 1982). The portion of the outermost bilayer of the liposome that is still immersed in water will have $\gamma \approx 0$. In the interface a pressure difference will therefore exist across the line of contact of the liposome particle with the air/water surface and act to shear the outermost bilayer along the line of contact. The outermost bilayer would be severed if this shearing force is greater than the cohesive forces within the bilayer; the severed bilayer fragment would then be free to spread on the air-water surface. Since the Laplace pressure across a bilayer at an air-water surface should be present at all temperatures, only because bilayer weakening has occurred at T* does the interface shear mechanism come into effect.

Table. Comparison of critical temperature, T*, for total lipid extracts from normal biomembranes with physiological temperature of the original membrane, Tp

Membrane lipid source	T*, °C	Tp[‡], °C
Human red blood cells	37.2	37.0[a]
Rabbit red blood cells	39.0	38.6–40.1 (Kozma et al., 1974)[a]
Rabbit cerebellum	39.4	
Rat spinal cord	39.0	35.8–37.6 (Bivin et al., 1979)[a]
Rat cerebrum	40.0	38.4–39.0 (Obermeyer et al., 1991)[b]

T* measurements were reproducible ± 0.2°C. No attempt was made to separate the different types of membrane present in the neural tissues studied. T* for a mixture of the total lipids from all the membranes in a tissue will, in principle, equal values obtained for extracts from each individual membrane present (Gershfeld, 1989*b;* Ginsberg et al., 1991).
[‡] Tp values listed are: [a]rectal temperatures, and [b]hypothalamic temperatures.

This model for surface bilayer formation also explains why the critical unilamellar state forms much more rapidly in the surface (within several minutes; Tajima & Gershfeld, 1985) compared to its rate of formation in the dispersion (many days; Gershfeld et al., 1993) where the surface forces are absent. Accelerating the rate of the topological transformation to ULVs will, however, occur in the suspension when a shearing stress is imposed by the rotating disk.

The thermodynamic properties of the lipids at T* have shown that the surface bilayer and the critical unilamellar state of the dispersion are equivalent structures (Gershfeld, 1989*b*). Our shear stress studies provide independent evidence that the critical unilamellar state in the surface is a manifestation of processes which occur spontaneously in the bulk dispersion. The formation of the critical bilayer state in the air-water surface is likely to be accelerated by surface forces. In the bulk dispersion, the MLV-ULV transformation in the absence of these surface forces is extremely slow; given sufficient time at T*, the same unilamellar state would form.

That bilayer weakening occurs only within a narrow temperature range around T* provides further evidence that a unique configuration of bilayer lipids exists that is manifested in the unilamellar state at T*. Since these conditions occur at a singularity in temperature, exhibit a heat capacity anomaly, and a slowing-down of the MLV-ULV transformation (Gershfeld et al., 1993) all characteristics of a critical point (Sengers, 1994), we have concluded that the unilamellar state at T* and the membrane bilayer are critical states. Although we do not as yet have a molecular description, bilayer weakening restricted to the narrow temperature region of T* is a reflection of the uniqueness of the molecular interactions in the critical bilayer state. How these interactions affect specific processes in membranes will be subjects of further study.

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