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A Singular State of Membrane Lipids at Cell Growth Temperatures[†]

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ABSTRACT: Cells adjust their membrane lipid composition when they adapt to grow at different temperatures. The consequences of this adjustment for membrane properties and functions are not well understood. Our report shows that the temperature dependence of the diffusion of a probe molecule in multilayers formed from total lipid extracts of *E. coli* has an anomalous maximum at a temperature corresponding to the growth temperature of each bacterial preparation (25, 29, and 32 °C). This increase in the lateral diffusion coefficient, *D*, is characteristic of membrane lipids in a critical state, for which large fluctuations of molecular area in the plane of the bilayer are expected. Therefore, changes in lipid composition may be due to a requirement that cells maintain their membranes in a state where molecular interactions and reaction rates are readily modulated by small, local perturbations of membrane organization.

Cells adjust their membrane lipid composition in response to changes in ambient temperature (*1*). This behavior has led to suggestions that membrane lipids at physiological temperatures are in a unique state; when confronted with changing ambient conditions, a cell somehow senses the physical structure of this state and activates the necessary biochemical reactions to maintain it (*2*). Yet, the most studied global physical property of membrane lipids, viscosity, or "fluidity", is relatively insensitive to changes in ambient temperature or membrane composition. Furthermore, many membrane enzymes are insensitive to changes in membrane lipid viscosity (*3*). This suggests either that cells are adjusting local composition to maximize enzyme function, or that some other, undefined, global property of membrane lipids is altered by changing temperature. By measuring the diffusion of a fluorescent-labeled phospholipid molecule as a probe, we demonstrate that, at the temperature of cell growth T_g, membrane bilayers exhibit special structural properties that are very sensitive to small changes in temperature. It follows from thermodynamic arguments (*4*) that membrane structure and, consequently, membrane function can be modified by relatively small changes in lipid and protein composition when the temperature is close to T_g.

Our measurements were prompted by studies of the aqueous dispersions of phospholipids that suggested the presence of critical phenomena in synthetic fluid bilayers. These studies indicated that a transformation between two multilamellar liquid-crystalline phases occurs over a narrow temperature range (*4*, *5*), characterized by formation of an intermediate unilamellar state at a temperature T^* that is unique for each lipid composition (*6*). This intermediate state exhibits a heat capacity anomaly and an equilibration time that increases proportionally with $(T - T^*)^{-1}$ (*5*). Moreover, lipid vesicles show increased fragility and unusual mechanical properties at T^* (*7*). Of more biological significance, the total lipid extracts of membranes, obtained from a variety of cells whose growth temperatures T_g span a wide range, form the putative critical state in vitro at a T^* that is, within experimental error, equal to T_g (*7*, *8*).

Since diffusion coefficients in fluids show characteristic behaviors at critical points (*9*), we used the technique of fluorescent recovery after photobleaching (FRAP)[1] (*10*) to gain further insights into these observations, as well as into their possible biological consequences. Our studies involved measuring the diffusion coefficient of a fluorescent phospholipid probe, NBD-PE, in four different bilayer preparations. Three were the total lipid extracts of *E. coli* grown at 25, 29, and 32 ± 0.5 °C. The fourth sample was pure dimyristoylphosphatidylcholine (DMPC) whose critical temperature, $T^* = 29$ °C, had been previously determined (*4*−*6*); the behavior of this lipid served as a guide for our studies of the complex lipid mixtures obtained from *E. coli*.

MATERIALS AND METHODS

Packed cells of *E. coli*, strain BL21, cultured at 25, 29, and 32 °C and harvested during log-phase growth, were provided by Dr. J. Shiloach, NIDDK, NIH. The temperature control of the fermenter was ±0.5 °C. Membrane lipids were

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[1] Abbreviations: FRAP, fluorescence recovery after photobleaching; DMPC, dimyristoylphosphatidylcholine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; CL, cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine.

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extracted by the method of Rose and Oklander (*11*), and total phospholipid concentrations of the final chloroform solutions were obtained by phosphorus analysis (*12*). These lipids were stored as chloroform solutions under nitrogen at −20 °C. Membrane lipids were separated into their lipid classes by thin-layer chromatography using plates of silica gel G and a solvent system of chloroform/methanol/water (65:25:4 by volume). The spots of each lipid class were visualized by spraying the plates with Rhodamine 6G, and viewing under UV. The spots were scraped and collected, and their phosphorus content was assayed (*12*). DMPC (Avanti Polar Lipids, Inc., Alabaster, AL), which yields a single spot by thin-layer chromatography (greater than 99% purity), was used as supplied.

Specimens were prepared for FRAP measurements by the following procedure: 50 μL of a solution of phospholipid and NBD-PE (Molecular Probes, Inc., Eugene, OR) 0.5 mol % in chloroform/methanol (2:1 v/v) is deposited on a carefully cleaned microscope coverslip and dried for approximately 2 h under a gentle stream of nitrogen. The lipid-coated coverslip then is placed on a microscope slide having a concave surface previously filled with distilled water to overflowing. Excess water is removed by wicking with absorbent paper, and the edges of the coverslip are sealed with a silicone rubber sealant (RTV-108, General Electric Co., Waterford, NY). The sealed slides containing multilamellar vesicles (MLVs), some adhering to the coverslip, are ready for study 24 h after their preparation. Generally, a smooth 100−2500 μm^2 area of flat film is used for the FRAP measurements. Specimen temperature is maintained to ±0.01 °C over a 15 °C range above ambient temperature by resistive heaters and a thermocouple that are attached to the slide, using a controller designed and built by Dr. C. P. Mudd; the variation in temperature across the sample is estimated to be no greater than a few tenths of a degree.

FRAP measurements were made using spot photobleaching (*10*). The spot radius was approximately 2.5 μm, but varied slightly from day to day that resulted in minor variations in *D*. However, the spot radius was constant during each temperature scan experiment that often took 10−12 h to complete. We found in this study that it is difficult to control the depth of bleaching, and that there is a correlation between bleach depth and *D*, viz., $D \approx D_{50} \exp[0.022(B - 50)]$, where *B* is the bleach depth as a percent of the initial signal. For purposes of comparison, all diffusion coefficients were corrected to equivalent values at 50% bleach. In this way, standard errors of the mean value determined for the 10−20 measurements that were made at each temperature were reduced to approximately ±5%. The utility of empirical calibrations in FRAP measurements is discussed by N. Perisamy and A. S. Verkman (*13*).

RESULTS AND DISCUSSION

Figure 1 shows the diffusion coefficient *D* of the probe NBD-PE as a function of temperature in DMPC multibilayer films. *D* increases with temperature to a peak at 28.6 °C, followed by an abrupt decrease as *T* is raised further. The temperature corresponding to the maximum value of *D* coincides with *T** (*4*−*6*). *T** is appreciably higher than the gel−liquid-crystalline transition temperature ($T_m \approx 23$ °C), which is marked by a dramatic increase in *D* as T_m is exceeded (*14*). Above T_m the bilayers are fluidlike, so *D* should increase only gradually with temperature. The appearance of a maximum in *D* at *T**, therefore, suggests that another structural change takes place at this temperature. This deduction is consistent with previous observations that a transformation between two multilamellar liquid-crystalline bilayer states occurs at *T** (*5*).

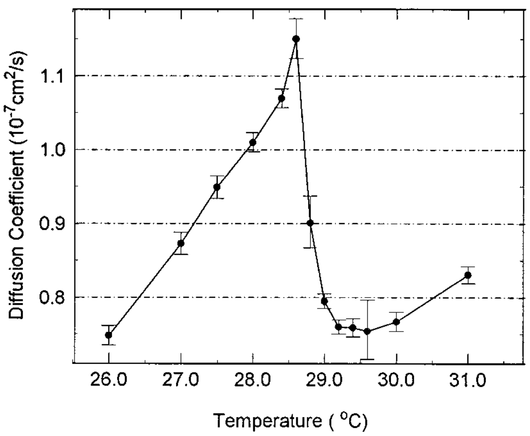

FIGURE 1: Diffusion coefficient *D* of the fluorescent probe NBD-PE moving in multilamellar DMPC films as a function of temperature. The points at each temperature represent the mean (±SD) of 10−20 independent measurements. Each measured value of *D* is obtained by employing an empirical relation that corrects for an experimentally observed correlation between the bleach depth and apparent diffusion coefficient (see Materials and Methods).

The observed diffusion of NBD-PE appears to be representative of a system in a critical state at *T**. Similar probe diffusion in the neighborhood of a critical point has been observed in several studies of "fragile" glass-forming liquids (*15*), in a study of diffusion at the critical mixing temperature of two organic liquids (*9*), and in investigations of surface monolayer diffusion (*16*). In each of these systems, as in the liquid-crystalline bilayers of DMPC, the diffusion coefficient of the probe is maximal at the critical temperature. Thus, the peak in *D* observed in DMPC bilayers at *T** appears to be a phenomenon characteristic of probe diffusion at fluid−fluid phase transitions. Since fluids at critical points generally exhibit large fluctuations in density, additional evidence that, at *T**, DMPC is at a critical point lies in the bilayer compressibility modulus which shows an unexpected dip as *T* approaches *T** (*17*). This behavior can be ascribed to an increase in fluctuations. A measure of these fluctuations may be derived from the bilayer area compressibility, κ_A, which is given by the relation (*18*): $\kappa_A = \sigma_A^2/(\langle A\rangle_T k_B T)$, where $\sigma_A^2 = \langle A^2\rangle_T - \langle A\rangle_T^2$ represents the mean square fluctuations in bilayer area about the mean value $\langle A\rangle_T$. Using the Needham and Evans (*19*) value for κ_A for DMPC at *T**, we find that the fluctuation in area is approximately 20% of the average area. Such large density fluctuations, which may indicate dynamic domains of differing sizes, must be related to the observed enhancement in *D*. The fluctuations at *T**, the critical point of fluid−fluid transitions, are physically distinct from those proposed to occur at T_m where gel (solid) and fluid phases coexist (*20*).

Measuring probe diffusion in multibilayers formed by the membrane lipids of the three *E. coli* preparations, we found the same signature as we had in DMPC multibilayers, but at different temperatures for each bacterial lipid preparation. Figure 2 shows *D* as a function of temperature for the total

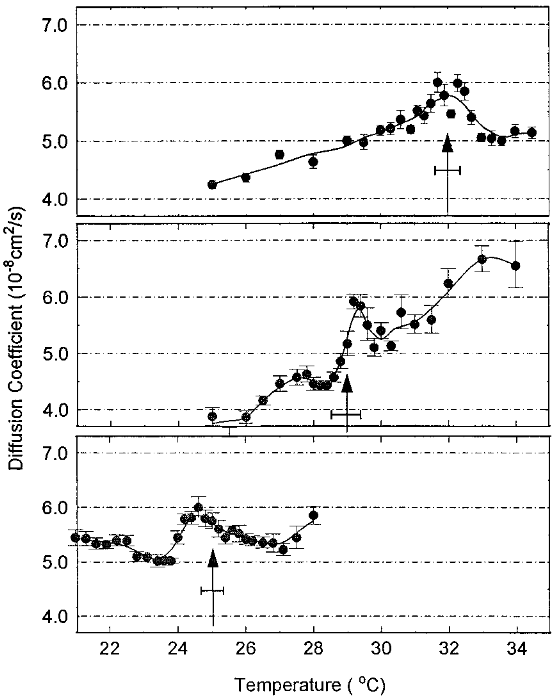

FIGURE 2: Diffusion coefficient D of the fluorescent probe NBD-PE as a function of temperature in multilamellar films of the total membrane lipids obtained from *E. coli* preparations grown at 25 °C (bottom), 29 °C (middle), and 32 °C (top). The growth temperatures are indicated by the arrows, and have an uncertainty of ±0.5 °C. Each point is the mean (±SD) of 10−20 independent measurements corrected by an experimentally observed correlation between the bleach depth and apparent diffusion coefficient (see Materials and Methods). Since the spot radius varied slightly from day-to-day, the values of D at the peaks were taken as 6.0×10^{-8} cm^2/s (a typical value), and all related measurements were scaled to this value.

Table 1: Phospholipid Composition (mol %, ±SD) of *E. coli* Membranes as a Function of Growth Temperature (T_g)

	T_g (°C)		
lipid	25 ± 0.5	29 ± 0.5	32 ± 0.5
PS	3.5 ± 0.3 (n = 4)[a]	3.3 ± 0.6 (n = 10)	4.5 ± 0.6 (n = 9)
PG	13.9 ± 1.1	14.6 ± 1.8	10.6 ± 1.0
PE	78.3 ± 1.7	76.8 ± 2.0	78.3 ± 2.0
CL	3.3 ± 0.3	3.6 ± 0.8	5.4 ± 0.4

[a] n is the number of measurements.

membrane lipids of the three samples obtained from cells grown at 25, 29, and 32 °C. Within experimental error, the maximum value of D occurred at a T^* that equaled the growth temperature, T_g , of the bacteria. Since $T^* = T_g$ for bilayers formed from total membrane lipid extracts, we deduce that the membranes of these cells are in a critical state at their growth temperatures (*4*, *8*).

The cell membrane lipid composition varied slightly with T_g (Table 1). The fatty acid composition of this strain of *E. coli* also undergoes a modest change in composition over this temperature range (*21*). While many phase transitions broaden when several components are present, the peak in each of these multicomponent lipid preparations occurred over a narrow temperature range (Figure 2). Thus, the presence of a large number of lipid components did not significantly affect the mechanism leading to the diffusion peak, indicative of the special character of this system. Another aspect of this state is that only small changes in lipid composition are sufficient to change the critical temperature of the membrane.

The correspondence between T^* and T_g has been observed for a wide range of cell types (e.g., RBC's, bacteria, squid nerve, mammalian brain tissue), whose physiological temperatures vary over the range 10−60 °C (*7*, *8*, *22*). Thus, we believe that membrane bilayers are in a fluidlike critical state at, or very near, their physiological growth temperatures, and that cells strive to maintain this state by changing their lipid compositions. A membrane that is maintained at or near a fluid critical point can be a significant advantage to a cell. Changes in local lipid composition, charge, or mechanical stress can shift bilayers to a state away from criticality, altering diffusion rates of embedded macromolecules, thereby influencing myriad membrane-linked reactions. Living cells are in a perpetual state of dynamic biochemical activity, so a variation in reaction rates in turn can have global effects on cell behavior. Critical conditions in membrane bilayers may also influence the interactions among the embedded proteins. The finding of a critical state at physiological growth temperatures provides a long-sought bridge between the physical properties of lipids and their role in membrane biology.

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