PHOSPHOLIPID SURFACE BILAYERS AT THE AIR-WATER INTERFACE

III. Relation Between Surface Bilayer Formation and Lipid Bilayer Assembly in Cell Membranes

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ABSTRACT Lipid bilayer assembly in cell membranes has been simulated with total lipid extracts from human red blood cells and from mesophilic and thermophilic bacteria grown at several temperatures. Aqueous dispersions of these natural lipid mixtures form surface bilayers, a single bimolecular lipid state, but only at the growth temperature of the source organism. Thus, a single isolated bilayer state forms spontaneously in vitro from lipids that are available in vivo at the growth temperature of the cell. Surface bilayers form at a specific temperature that is a function of hydrocarbon chain length and degree of fatty acid unsaturation of the phospholipids; this property is proposed as an essential element in the control of membrane lipid composition.

INTRODUCTION

The surface bilayer is a single bimolecular lipid layer that forms spontaneously at the air-water surface of phospholipid dispersions (Gershfeld and Tajima, 1979; Tajima and Gershfeld, 1985; Ginsberg and Gershfeld, 1985). In contrast to other lipid bilayer states that are readily prepared over a wide range of temperatures, surface bilayers form only at a specific temperature. Using pure phospholipids to characterize this critical temperature, it has been shown to be a function of hydrocarbon chain length, degree of fatty acid unsaturation, and with simple mixtures of phospholipids, a function of composition (Tajima and Gershfeld, 1985).

Whether more complex phospholipid systems would also form surface bilayers has now been examined with the total lipid extracts from red blood cells and from two bacterial species grown at several temperatures. These particular bacterial systems were chosen because they grow over a wide range of temperatures, thereby providing a stringent examination of the relationship between the growth temperature of the cells and the temperature of surface bilayer formation of the extracted membrane lipids.

The temperature at which surface bilayers form can be identified from surface pressure measurements and with radiotracers[1] (Gershfeld and Tajima, 1979; Tajima and Gershfeld, 1985), or by permeability studies (Ginsberg and Gershfeld, 1985). Each method gives identical temperatures. Here the surface pressure method has been used exclusively.

The use of the surface pressure method may also be justified by a thermodynamic analysis of the system (Tajima and Gershfeld, 1985). Surface bilayers have been shown to form at the temperature where the surface pressure of the dispersion is a maximum. Two important properties of the system appear uniquely at the surface pressure maximum: (*a*) the composition of the surface film becomes identical with that of the bulk dispersion; and (*b*) the partial molar entropy of each surface component becomes identical with that of each of the components of the bulk dispersion. Thus, when the bulk dispersion is a liquid crystal consisting of lamellar bilayers the surface would, of necessity, also contain a bilayer. This thermodynamic argument is the underlying basis for utilizing the surface pressure maximum for identifying the temperature of surface bilayer formation. The analysis has been verified

[1]Although other radiotracer studies report that vesicles may be present in phospholipid surface films (Pattus et al., 1978; Schindler, 1979), their presence may be accounted for by the method used for measuring film concentrations. The method employed in those studies entails transferring the film from the surface of the dispersion to a lipid-free solution. However, this procedure also transfers a significant amount of the underlying fluid — a region ~0.01–0.02 cm deep (Schulman and Teorell, 1938; Pak and Gershfeld, 1967). Thus, some of the dispersion will be transferred along with the film. In contrast, surface bilayers were measured by an in situ method; moreover, the amount of lipid adsorbed at equilibrium was found to be independent of the lipid concentration in the dispersion (Tajima and Gershfeld, 1985).

with binary lipid mixtures of phospholipids (Tajima and Gershfeld, 1985), and is assumed to be applicable to the multicomponent dispersions of membrane lipids used here.

The following data show that each of the mixtures of cell membrane lipids forms a surface bilayer, as indicated by a maximum in the surface pressure-temperature phase diagram of the aqueous dispersion. However, the surface bilayers of these complex lipid mixtures form only at the temperatures where the source organisms were grown. This striking result suggests that a relation exists between surface bilayer formation and lipid bilayer assembly in cell membranes. A discussion of this relationship within the framework of the thermodynamic properties of the surface bilayer system is presented; the relationship entails formation of a critical state in which single lipid bilayers form throughout the bulk aqueous phase. The study concludes with a discussion of how the critical state may be utilized by the cell for controlling the composition of its membrane lipids.

METHODS

Preparation of Membrane Lipids

The following cellular systems were examined: *Bacillus stearothermophilus* (*B. stearothermophilus*; ATCC No. 12016), grown at 49° and 59°C, using a minimum glucose growth medium (Souza et al., 1974); *Escherichia coli* (*E. coli*; ATCC No. 11303) grown at 20° and 30°C, using medium C (Roberts et al., 1955); a pooled sample of human red blood cells was separated from freshly collected whole blood. All bacteria were harvested during exponential growth; bacterial fermentation temperatures were controlled ±1°C. Lipids were completely extracted from the cells (Rose and Oklander, 1965), and the extract was dried under nitrogen. The inner and outer membranes of *E. coli* were not separated, so that the total lipid from both membranes is contained in the extract. The lipids were stored under nitrogen at −20°C as chloroform solutions in screw-topped test tubes wrapped in aluminum foil. The lipids were used within one week of their preparation.

Surface Pressure-temperature Phase Diagrams

Surface pressures were measured with the horizontal float film balance, using the equilibrium spreading pressure method (Gaines, 1966; Gershfeld, 1976). This method entails placing bulk lipid directly on the water surface in an amount that is much larger than is necessary to form a condensed monolayer of the lipid. The equilibrium spreading pressure has been shown to be identical to the surface pressure obtained for dilute dispersions of phospholipids using the Wilhelmy plate technique (Gershfeld and Tajima, 1979; Tajima and Gershfeld, 1985). The horizontal float system was employed because it minimizes contact-angle hysteresis that is occasionally encountered with the Wilhelmy plate (Gaines, 1966). All components of the film balance that are in contact with water—trough and float—were made of Teflon. In preliminary studies of the phospholipid spreading pressures, film leakage past the conventional rigid barriers was encountered. The conventional movable barrier was therefore replaced by a ribbon of Teflon (width 0.5 in, length 12 in, 0.004 in thick) that was made continuous with each of the fixed ends of the float's end-loops; the Teflon ribbon effectively acted as a flexible wall, replacing both the rigid wall of the trough and the movable barrier. The lipid film was thus confined within an area surrounded by a continuous loop of Teflon; the film area could be changed by pushing the free surface of the Teflon loop either towards the float, to decrease the film area, or away from the float to increase the area. The film area could easily be changed by moving a conventional barrier against the flexible Teflon ribbon; remote manipulation of the barrier permitted control of the film area external to the insulated walls of the box surrounding the film balance.

The entire system was maintained under an atmosphere of nitrogen (>99%), and near 100% relative humidity by bubbling the gas through water at the same temperature as the trough. The nitrogen atmosphere was necessary to prevent oxidation of the lipid film; adding antioxidants to the water in the trough was ineffective for preventing oxidation of the lipid films.

Temperature control of the film balance was maintained by circulating constant temperature water through copper tubing located in the base of the trough and in the walls of the thermally insulated box surrounding the film balance. Temperatures of the water surface and the surrounding nitrogen phase were monitored by thermistors; the two temperatures were generally within 0.5° of each other, except at the very highest temperatures attainable (60°C) where the difference was ~1°. The reported temperatures are those for the aqueous surface.

The dried membrane lipid preparations were generally in the form of pastes. The lipid was deposited on clean, disposable glass loops, and then transferred to the water surface through a small opening in a wall of the thermally insulated box covering the film balance. The entire film balance system was equilibrated before adding the lipid.

To establish that equilibrium conditions were attained, the final surface pressure was shown to be constant with time and independent of the film area and of the amount of lipid added. A typical procedure for testing whether a film had reached the equilibrium spreading pressure was as follows: the film area was reduced, thereby causing first a transient increase in the surface pressure, followed by a spontaneous, gradual decrease of the surface pressure to the equilibrium value; the area of the film was then expanded causing a transient decrease in surface pressure that was followed immediately by a spontaneous slow increase of the surface pressure to the equilibrium value. This procedure was repeated at least twice; the precision for the equilibrium spreading pressures measured by this procedure was ±0.3 dyn/cm. Temperature control was maintained at ±0.1°C.

The physical state of lipid in the dispersion was determined by calorimetry, using a differential scanning calorimeter (model DSC 2; Perkin-Elmer Inc., Norwalk, CT). In addition, a phase contrast microscope equipped with a constant temperature stage provided a description of the morphological state of the dispersed lipid; temperature control for the stage was ±0.2°C.

RESULTS

Previous studies with phospholipid dispersions indicated that surface bilayer formation occurred when the dispersion contained a single, liquid crystalline phase (Gershfeld and Tajima, 1979; Tajima and Gershfeld, 1985). To establish that this condition prevails in the multicomponent membrane lipid dispersions, the physical states of the dispersions formed by the membrane lipid extracts were determined by differential scanning calorimetry and phase contrast microscopy.

The gel-liquid crystal transitions for the aqueous dispersions of the bacterial lipids are broad, spanning a temperature range of 30–40°C. For *E. coli* grown at 20° and 30°C, the endotherms were completed at 13° and 20°C, respectively; for *B. stearothermophilus* grown at 49° and 59°C, the final endotherm temperature was 39°C for both preparations. The liquid crystal state for these dispersions was

confirmed by phase contrast microscopy; liposomes, and thinwalled vesicles were the predominant structures in the dispersions. The gel-liquid crystal transition for aqueous dispersions of the red blood cell lipids was not detected by differential scanning calorimetry. However, for temperatures exceeding −20°C, x-ray diffraction studies indicate that the dispersions form a single liquid crystalline phase (Gottleib and Eanes, 1974). Moreover, in the temperature interval of 20°–50°C, the liquid crystalline state for the red blood cell lipid dispersions was confirmed by the appearance of vesicles, liposomes, and myelin figures under phase contrast microscopy. In general, for the cellular systems in this study, dispersions of the membrane lipids form a single liquid crystalline phase a minimum of 7°–10°C below the growth temperature of the parent cell.

The surface pressure-temperature phase diagrams for the lipids extracted from the five cell preparations are given in Fig. 1. In four of the five systems studied, a surface pressure maximum was observed; in the fifth system, *B. stearothermophilus* at 59°C, temperature control for surface pressure measurements exceeding 60°C was not attainable. Nonetheless, there is a good indication of a surface pressure maximum near 60°C. The broadened surface pressure maxima for these multicomponent systems was expected, because earlier studies with simpler lipid mixtures indicate a broadening of the maximum with increase of the number of components (Tajima and Gershfeld, 1985).

DISCUSSION

The principal objectives of this study were to establish that multicomponent lipid mixtures from cell membranes would form surface bilayers, and to test whether a relationship exists between cell growth temperatures and the temperature of surface bilayer formation. The latter objective was pursued because the temperature of surface bilayer formation varies with lipid composition in a manner that resembles the way that the phospholipid fatty acid composition of many biological membranes varies with cell growth temperature. Thus, for *E. coli* (Marr and Ingraham, 1962; Shaw and Ingraham, 1962) and for *B. stearothermophilus* (Souza et al., 1974; Reizer et al., 1985) increasing the growth temperature enriches the saturated fatty acid content of the membrane lipids. Correspondingly, the temperature of surface bilayer formation increases when unsaturated fatty acid moieties of phospholipids are replaced by saturated acyl chains (Tajima and Gershfeld, 1985).

The necessary conditions for the formation of surface bilayers exist when the lipid phase of the dispersion is in the liquid crystalline lamellar bilayer state, and the surface pressure-temperature phase diagram shows a maximum. Fig. 1 indicates that each of the five lipid mixtures exhibits a surface pressure maximum; the temperature of each maximum ranges from 7° to 20°C above the gel-liquid crystal transition temperature of the respective equilibrium dispersion. Thus, at the surface pressure maximum the conditions for surface bilayer formation have been attained by each of the membrane lipid dispersions.

The relationship between surface bilayer temperatures and growth temperatures of the bacterial cells and erythrocytes is presented in Fig. 2; agreement between the two for each system is within the limits of temperature control of cell growth. As the surface bilayer is an equilibrium system, defined by the temperature of formation and the lipid composition, this excellent agreement is thermody-

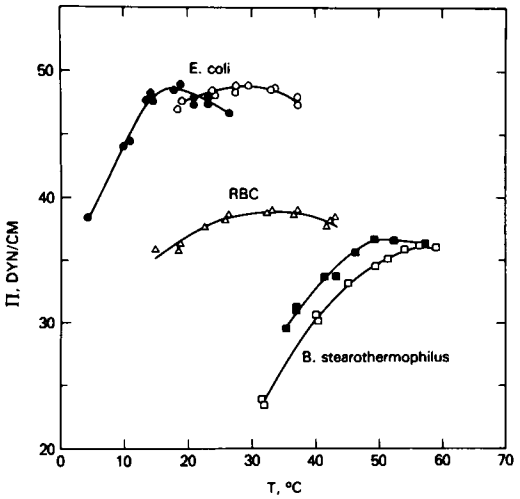

FIGURE 1 Surface pressure (π)-temperature phase diagrams for membrane lipids extracted from: *E. coli*, grown at 20°C(●), 30°C(○); *B. stearothermophilus*, grown at 49°C (■), 59°C(□); human red blood cells (△).

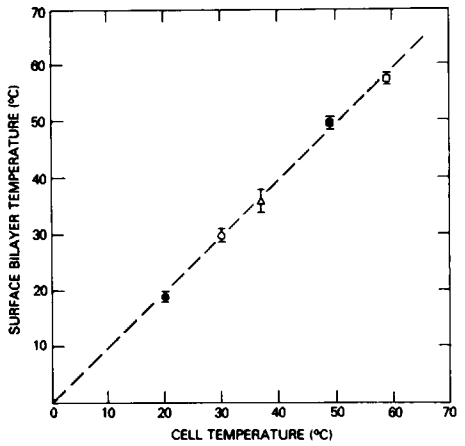

FIGURE 2 Comparison of cell temperatures and temperature of surface bilayer formation for extracts of membrane lipids. Cell temperatures refer to growth temperatures for the bacteria, and body temperature (taken to be 37°C) for erythrocytes. Surface bilayer temperatures are obtained from the surface pressure maxima in Fig. 1; symbols are the same as in Fig. 1. Surface bilayer temperatures are given as ranges to reflect the breadth of the surface pressure maxima. The dotted line indicates 1:1 relation between the two temperatures.

namically consistent with the existence of the surface bilayer in each of the cell membranes at their growth temperatures.

Recognizing that surface bilayers form at an air-water interface, are these results relevant to the cell? One interpretation rests on the thermodynamic properties of the surface bilayer system. At the surface pressure maximum (where surface bilayers form) the composition and the partial molar entropies of the components in the surface bilayer are identical with the composition and partial molar entropies of the bulk lipid in the dispersion (Tajima and Gershfeld, 1985). These properties signify the formation of a critical state in which the surface and bulk lipid states become indistinguishable. It follows that at the critical temperature of surface bilayer formation, the bulk lipid state becomes identical with the surface bilayer, and single, isolated bilayers will form throughout the entire system, with the bulk dispersion presumably consisting of unilamellar vesicles. Furthermore, this analysis indicates that single bilayers will form whenever the requisite equilibrium conditions are present, even when the system is devoid of an air-water surface. Thus, when the temperature and lipid composition of cell membranes satisfy the requirements for surface bilayer formation it may be surmised that single bilayers will form spontaneously in the membrane.

The deduction that single bilayer vesicles form at the surface bilayer critical temperature is seemingly paradoxical because it is commonly observed that liquid crystalline phospholipid dispersions form multibilayer structures over a broad range of temperatures. Indeed, the phase contrast microscopy studies of each of the five membrane lipid preparations indicate that liposomes and vesicles are present over a wide range of temperatures, including the point at which the surface bilayer forms. Some of the elements for resolving this apparent paradox are included in the results of recent studies with very dilute phospholipid dispersions: when dispersions at concentrations near the solubility limit of the lipid are used, single bilayer vesicles appear at the same temperature where the surface bilayer forms; at temperatures above and below the surface bilayer temperature nonunilamellar states form (Gershfeld et al., 1986). The formation of surface bilayers may therefore be taken as evidence for the formation of single bilayers in phospholipid dispersions.

The surface bilayer state fulfills several important requirements for a satisfactory model of membrane bilayer assembly: (*a*) it is unilamellar; and (*b*) it forms spontaneously from lipids that are available in the metabolic pool at the growth temperature of the cell.

The surface bilayer model also helps to explain why cell membrane lipids are modified when the growth temperature of the cell is changed. Because bilayer composition influences the temperature of surface bilayer formation, a change in the environmental temperature of the cell requires a corresponding change in the lipid composition if new membrane bilayers are to form. The biochemical transformations that occur among the membrane phospholipids are the cell's response to the new temperature condition for bilayer formation in the membrane. This model also suggests that when environmental temperature is altered, the membrane signals its need for new lipid, perhaps by disruption or reorganization of the existing unilamellar structure; metabolic processes might then be stimulated to produce the necessary phospholipid transformations. How the response is triggered is, of course, an open question.

In summary, a single isolated bilayer state forms spontaneously in vitro from lipids that are available in vivo at the growth temperature of the cell. Single bilayers, as monitored by the surface bilayer state, form at a single critical temperature; this property is proposed as an essential feature in the control of membrane lipid biogenesis. Among other questions remaining and currently under study are the influence of lipid phase relations on surface bilayer formation, and whether the surface bilayer state forms with lipids from other types of cell membranes (e.g., endoplasmic reticulum, nuclear membranes, etc.).

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