Intrinsic curvature hypothesis for biomembrane lipid composition: A role for nonbilayer lipids

(H_II phase/lipid phase transitions/phospholipid)

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ABSTRACT A rationale is presented for the mix of “bilayer” and “nonbilayer” lipids, which occurs in biomembranes. A theory for the L_{α}-H_{II} phase transition and experimental tests of the theory are reviewed. It is suggested that the phase behavior is largely the result of a competition between the tendency for certain lipid monolayers to curl and the hydrocarbon packing strains that result. The tendency to curl is quantitatively given by the intrinsic radius of curvature, R_{s}, which minimizes the bending energy of a lipid monolayer. When bilayer (large R_{s}) and nonbilayer (small R_{s}) lipids are properly mixed, the resulting layer has a value of R_{s} that is at the critical edge of bilayer stability. In this case, bilayers may be destabilized by the protein-mediated introduction of hydrophobic molecules, such as dolichol. An x-ray diffraction investigation of the effect of dolichol on such a lipid mixture is described. This leads to the hypothesis that biomembranes homeostatically adjust their intrinsic curvatures to fall into an optimum range. Experimental strategies for testing the hypothesis are outlined.

All biological membranes contain a great diversity of lipids (1). As Raetz (2) has succinctly noted, “The metabolic control and biological significance of lipid heterogeneity are not well understood. Several fundamental questions remain unresolved in this area (and in all organisms). These include the following: (1) What mechanisms regulate (or set) the total membrane phospholipid content of cells? (2) What regulates the ratios of polar headgroups and fatty acid species? . . . (6) What are the functions of the individual phospholipid species?” Our lack of understanding is poignantly illustrated by experiments with bacteria in which the fatty acid synthesis pathways have been genetically or chemically disabled (3, 4). Thus, these bacteria are dependent on fatty acids provided in the nutrient medium. If the available fatty acid mix is changed, the cells respond by complicated readjustments of the fatty acids incorporated into the membranes (4). The cells appear to be juggling the membrane lipid composition to seek homeostatic control of some crucial membrane quantities. We do not know what these quantities are.

It recently has been recognized that a large fraction of the lipids of many biological membranes form the liquid-crystalline H_{II} phase (refs. 5 and 6; Fig. 1) when purified and hydrated under physiological conditions (7). These are sometimes referred to as “nonbilayer” lipids. The remaining, so-called “bilayer,” lipids primarily form bilayers when purified and hydrated under the same conditions. This has led to a lively debate as to whether nonbilayer lipid structures are biologically important. Membrane lipids are in the bilayer form most of the time in the cell; therefore, it has been argued, the nonbilayer preference of certain purified lipids is a consequence of isolation of the lipid and is irrelevant to the mixed-lipid in vivo system. The counterargument has been that, although membranes are bilayers most of the time, they also must be capable of forming transient, nonbilayer structures because vital processes such as fusion, endocytosis, etc., are topologically impossible with intact bilayers. It has been suggested that the underlying molecular characteristics that cause certain lipids to form nonbilayer phases upon purification and interbilayer contact also enhance the formation of transient nonbilayer features in functioning biomembranes (7, 8).

In this article I review some recent theoretical and experimental results as to why certain lipids undergo the transition from stacked bilayer, or L_{α} (5, 6), to the liquid-crystalline H_{II} phase. It is postulated that a quantity called the monolayer intrinsic radius of curvature most fundamentally distinguishes the bilayer from the nonbilayer lipids. New experimental results are then presented on the phase-modulating behavior of naturally occurring dolichols as a further test of the theory. It is shown that the phase-modulating ability of dolichol is dependent on the lipid mix having a sufficiently small value of the intrinsic radius of curvature. This leads to the hypothesis that the intrinsic radius of curvature is a quantity that is homeostatically controlled in living biomembranes.

BACKGROUND

What underlying molecular characteristics enhance the formation of nonbilayer structures? The concept of molecular shape has been identified most often as the important characteristic: lipids with nonuniform (“cone-shaped”) average cross sections pack side-by-side into curved layers (7, 9). A difficulty with a literal formulation of the shape concept is that, above the chain melting temperature, lipids are highly flexible and do not have a specific shape. To illustrate, consider the average molecular volume occupied by a lipid in a pure system just below and above the temperature at which the system undergoes an L_{α}-to-H_{II} phase transition. Below the transition temperature, in the bilayer geometry, the average molecular area is uniform in cross section; above the transition temperature, it is tapered. In other words, the average molecular volume is, to a great degree, determined by the phase. The argument that the average molecular shape, in turn, determines the phase is then circular.

A more quantitative formulation of the shape concept would involve a determination of the free energy per molecule when it occupies a given molecular volume of a given shape. This was the approach used by Kirk et al. (10) in formulating a theory of the L_{α}-H_{II} phase transition. It was assumed that the shape-dependent free energy per molecule may be partitioned into components arising from elastic

Abbreviations: Ole_{2}-PtdCho, dioleoyl phosphatidylcholine; Ole_{2}-PtdEtN, dioleoyl phosphatidylethanolamine; PtdCho, phosphatidylcholine(s); PtdEtN, phosphatidylethanolamine(s); T_{αH}, transition temperature between L_{α} and H_{II} phases.
Bending of the lipid monolayers and hydrocarbon-packing energies. It was additionally necessary to include lattice-specific contributions, as, for instance, from hydration and electrostatic potentials.

Lipid monolayers (which form bilayers when placed tails-to-tails) are practically flat in the \( \text{L}_\alpha \) phase but are rolled into tight cylinders in the \( \text{H}_\Pi \) phase. The theory assumes that an elastic free energy, \( \mu_E \), is given to lowest significant order by

\[
\mu_E = k \left( 1/R - 1/R_e \right)^2.
\]

Here, \( k \) is an elastic constant and \( R \) is the radius of curvature of the lipid/water interface. We define the sign of \( R \) as positive for the \( \text{H}_\Pi \) phase and negative for the \( \text{H}_\upsilon \) phase (6). \( R_e \) is the "equilibrium," or "intrinsic," radius of curvature, which is defined self-consistently as the radius of curvature that minimizes \( \mu_E \). A lipid monolayer for which \( R = R_e \) is elastically relaxed. \( R_e \) is postulated to be a property of the lipid and does not vary appreciably with the lipid geometry. Factors that widen the splay of the tails (e.g., temperature or unsaturation) decrease \( R_e \). Factors which increase the effective headgroup area (e.g., charge of the headgroup) increase \( R_e \). If no other energies competed with \( \mu_E \), lipid monolayers would curl so that \( R = R_e \) to lower the elastic energy. We call the tendency for lipid monolayers to curl so as to lower \( \mu_E \) the expression of the intrinsic curvature.

Hydrocarbon-packing constraints were suggested as preventing the expression of large radii of curvature. We assume that, for a given lipid, temperature, and geometry, the lipid tails have a well-defined mean length that is the result of the number of gauche rotamer carbon bonds (11) excited in the hydrocarbon. For example, in the \( \text{H}_\Pi \) phase (Fig. 1), we take this length to be approximately equal to \( d_{\text{st}} \). Chains that are stretched beyond this length (i.e., to fill the stippled area of Fig. 1b) are in a high-energy state, much as stretched polymer strands. Furthermore, it is readily verified from the geometry (Fig. 1b) that the distance, \( d_{\text{max}} \), to the point in the stippled area furthest removed from a water cylinder is

\[
d_{\text{max}} = \frac{2}{\sqrt{3}} (R_e + d_{\text{st}}) - R_e,
\]

where \( R_e \) is the radius of the polar (crosshatched) core. Also, the ratio of the stippled area (stressed hydrocarbon) to the area of the lipid annulus (relaxed hydrocarbon) in the lattice (Fig. 1) is

\[
A = \frac{R_e + d_{\text{st}}}{2} \sin 60^\circ - \frac{\pi}{2} \left( \frac{(\pi/2)(2 R_e d_{\text{st}} + d_{\text{st}})}{2} \right).
\]

If \( d \) is kept constant, both \( d_{\text{max}} \) and \( A \) are seen to increase with \( R_e \). Thus, increasing the water-to-lipid ratio of the \( \text{H}_\Pi \) phase increases the hydrocarbon-packing free energy. In sum, this packing energy competes more effectively with the decrease in \( \mu_E \) as \( R \) increases toward large values of \( R_e \), i.e., the larger the radius of the water cylinder, the harder it is to pack the relaxed chains.

It is important to realize that an increase in the hydrocarbon-packing free energy will occur whenever the lipid tails are not all at the same mean relaxed length. This occurs whenever tails-to-tails lipid monolayers do not have parallel lipid/water interfaces. Thus, the competition between packing energy and \( \mu_E \) occurs for many curved structures, not just the \( \text{H}_\Pi \) phase. It is a general phenomenon associated with local expression of the intrinsic curvature.

If the amount of available water is limited, the lipid cylinders may be unable to expand to express large values of \( R_e \). However, in the presence of excess water, hydration repulsion (12) promotes separation of the headgroup surfaces (10). Likewise, electrostatic repulsion of charged lipid surf-

![Fig. 1. Schematic cross sections through seven unit cells of an \( \text{H}_\Pi \) lattice. (a) Lipid headgroups are represented by black dots attached to hydrophobic tails (wavy lines). The crosshatched areas represent water. The water-cored, lipid-coated cylinders extend out from the plane of the page. (b) \( \text{H}_\Pi \) phase is shown in a simpler schematic. Each annulus represents hydrocarbon at a nearly relaxed length, while the stippled areas are stressed hydrocarbon. The polar core has a radius, \( R_e \), while \( d_{\text{max}} \) is the maximally extended mean hydrocarbon length.](image)
approach zero for both lipids. But the lipid with a larger value of \( R_c \) would have a larger rise in hydrocarbon-packing energy. Thus, the lipid with a larger value of \( R_c \) would remain in the \( L_n \) phase to higher temperatures.

The picture of the phase transition, described above, can be tested by devising an experimental situation where the hydrocarbon-packing energy, which opposes the formation of nonbilayer structures, is removed. This can be done by adding a small quantity of a hydrophobic substance, such as a light oil, to the lipid. The oil can migrate in the hydrocarbon region and relax packing stress by preferentially partitioning into volumes where the chains are stressed, such as the stippled areas of Fig. 1b. In this case, expression of curvature is not accompanied by a large increase in packing free energy, and one expects that curved structures would be expressed at relatively low temperatures.

Experimental support of these ideas was obtained by Kirk and Gruner (13). The lipids dioleoyl phosphatidylethanolamine (Ole2-PtdEtn) and dioleoyl phosphatidylcholine (Ole2-PtdCho) were used. In excess water, both lipids were in the \( L_n \) phase at \(-5^\circ C\). However, whereas Ole2-PtdEtn had a \( L_n \)-to-\( H_n \) transition temperature \( (T_{BH}) \) between 5 and \(10^\circ C\), Ole2-PtdCho was still in the \( L_n \) phase even at \(80^\circ C\). It was first shown that the addition of a few percent oil to Ole2-PtdEtn dramatically lowered \( T_{BH} \). The lipid went into the \( H_n \) phase almost as soon as the temperature exceeded the chain melting (\( L_n \) to \( L_n \)) temperature. It was then shown that 3:1 Ole2-PtdEtn/Ole2-PtdCho mixtures have a \( T_{BH} \approx 50-55^\circ C \) without oil, but \( T_{BH} \) was reduced to \(-5 \) to \(0^\circ C\) when a few percent oil was added. Moreover, the lipid cylinders that resulted had water cores with a radius that was considerably larger than that for pure Ole2-PtdEtn \( H_n \) cylinders. The \( H_n \) dimensions increased further when the fraction of Ole2-PtdCho in the mixed system was increased.

These results may be interpreted by assuming that Ole2-PtdEtn had a small value of \( R_c \), whereas Ole2-PtdCho had a large value. The addition of oil removed most of the hydrocarbon packing stress and allowed the expression of curvature at dramatically low temperatures. \( H_n \) phases of Ole2-PtdEtn with oil had small cylinders because \( R_c \) was small for this lipid. When Ole2-PtdEtn and Ole2-PtdCho were mixed, the value of \( R_c \) for the mixture fell between the extremes of the pure components. Thus, the Ole2-PtdEtn/Ole2-PtdCho \( H_n \) phases had large cylinders, the radii of which increased with the fraction of Ole2-PtdCho. Most fundamentally, it appeared that Ole2-PtdEtn formed \( H_n \) phases at temperatures where Ole2-PtdCho was still in the \( L_n \) phase because \( R_c \) was smaller for Ole2-PtdEtn than for Ole2-PtdCho.

These experiments are highly suggestive of bilayer control mechanisms that may be active in living biomembranes. The dilemma faced by biomembranes is that they must be stable as bilayers most of the time but subject to disruption during localized events such as membrane fusion, endocytosis, cell fission, etc. What value of \( R_c \) is optimal for such behavior? If \( R_c \) were very small, then bilayers would be readily disrupted by the formation of curved structures because the hydrocarbon-packing energy, which opposes the formation of curved structures, is small for small radii of curvature. This is the case, for instance, for Ole2-PtdEtn. On the other hand, if \( R_c \) were large, then expression of curvature of the order \( R_c \approx R_c \) would engender a large hydrocarbon-packing energy. This makes bilayers very hard to disrupt, as is the case for Ole2-PtdCho. However, if \( R_c \) were maintained at intermediate values, then the competition between curvature and hydrocarbon packing would be such that bilayers are stable until hydrophobic molecules that affect the packing are introduced. This is the case with Ole2-PtdEtn/Ole2-PtdCho mixtures in the presence of oil. Protein-mediated introduction of hydrophobic molecules may be a mechanism for local disruption of bilayers. It was noted at the beginning of this paper that biomembranes appear to be a mix of bilayer (i.e., large \( R_c \)) and nonbilayer (i.e., small \( R_c \)) lipids. The mix may result in intermediate values of \( R_c \), which put the bilayer on the edge of stability.

As suggested by Kirk et al. (10), electrostatic and hydration contributions also affect the phase behavior. Bilayer stability is sensitive to these factors, especially when \( R_c \) is of intermediate values. Local modulation of the surface charge or surface hydration also may be mechanisms for the local destabilization of bilayers.

Do biomembranes contain hydrophobic molecules that release packing stress in a manner similar to the way oil affects Ole2-PtdEtn/Ole2-PtdCho mixtures? Consider, for example, the polyspernioid lipids known as dolichols. These long (=90 carbons) hydrocarbon lipids are necessary cofactors for the attachment of sugars to membrane proteins in the rough endoplasmic reticulum (14, 15). The sugars are synthesized outside the endoplasmic reticulum lumen, but the protein attachment site is inside the lumen. Dolichol is thought to be involved in the transfer of sugars across the endoplasmic reticulum membrane and the eventual attachment of the sugars to the proteins (16, 17). Neither the details of how the sugars are shuttled across the membrane nor the exact functional roles of dolichol are known.

Jensen and Schutzbach (18) used \(^{31}\)P NMR to investigate the effect of dolichol on PtdEtn/PtdCho mixtures. In the absence of dolichol, a 2:1 PtdEtn/PtdCho (39°C) mixture yielded the \(^{31}\)P-NMR spectrum expected from a predominantly bilayer organization. Upon the addition of dolichol, the spectrum was altered so that it was similar to that seen with lipids in the \( H_n \) phase. These authors also investigated the activity of mannosyltransferase II when reconstituted with various lipids. This membrane-bound enzyme catalyzes the transfer of GDP-mannose to a dolichol-linked oligosaccharide. The enzyme was optimally active above the lipid chain melting temperature when reconstituted in unsaturated PtdEtn and was relatively inactive when reconstituted in saturated PtdChos (19). When reconstituted in PtdEtn/PtdCho mixtures, the activity grew as the amount of PtdEtn increased (18). Jensen and Schutzbach (18) argued that the increased activity was associated with the tendency for the lipid to be in a nonbilayer form and not with the presence of PtdEtn because the enzyme was relatively inactive when reconstituted with the bilayer lipid dielaidoyl phosphatidylcholine \((T_{BH} = 60^\circ C)\). These studies are of interest both because of the effect of dolichol on PtdEtn/PtdCho mixtures and because of the possibility of an enzyme that is active in lipids that readily promote curved, or nonbilayer, structures.

I now report on the use of x-ray diffraction to further investigate the effect of dolichol on PtdEtn/PtdCho mixtures.

**METHODS**

Porcine liver dolichol in carbon tetrachloride (98% C\(_{20}-C_{18}\); Sigma) was vacuum dried and resuspended in chloroform; otherwise, procedures and material sources were as described (13). Briefly, Ole2-PtdEtn, Ole2-PtdCho, and dolichol, each dissolved in chloroform, were mixed in x-ray capillaries and dried under reduced pressures. Buffered saline solution was then added to excess to the lipid, and the capillaries were sealed and allowed to equilibrate at 5°C for at least 12 hr. X-ray diffraction was performed by using the Princeton SIV x-ray detector beam line (20, 21). The x-ray data were used to determine the phase behavior of the lipid at roughly 5°C intervals in an ascending temperature series from 0 to 30°C. The data to be shown indicate the lipid phase present and the basis vector lengths for each phase at each temperature. Also note that the 3:1 mix of lipid is nominal. The effect of mixing errors of several percent in the PtdEtn-to-PtdCho ratio is to shift vertically the \( H_n \) curve of Fig. 3.
For this reason, the absolute values of $H_{II}$ basis vector lengths should be considered to be accurate to only 2 Å.

**RESULTS**

As noted earlier, the phase behavior of 3:1 Ole$_2$-PtdEtn/Ole$_2$-PtdCho mixtures in excess water has been examined by Kirk and Gruner (13). Above 0°C, the mixture was in the L$_s$ phase with $T_{BH} = 50$–55°C. Upon the addition of 5% (wt/wt) dodecane, $T_{BH}$ was depressed below 0°C, and the lipid was in the H$_II$ phase to at least 85°C. Results of X-ray diffraction upon the addition of 2% (wt/wt) dolichol are shown in Fig. 2. At 0°C, the diffraction was dominated by a lamellar signature consisting of equally spaced Bragg orders (Fig. 2). The presence of a second phase was indicated by weak peaks associated with another lattice. As seen in Fig. 2, the rise in temperature was accompanied by a monotonic decrease in the intensity of the lamellar diffraction. Concomitantly, the intensity from the second lattice was seen to grow into a well-defined pattern with peak spacings in the ratio 1:2:3, indicative of hexagonal packing. This is interpreted as an L$_a$ phase, which predominated at low temperatures. As the temperature increased, more and more of the lipid was converted to an H$_II$ phase (Fig. 3).

The phase behavior of Ole$_2$-PtdCho with 2% dolichol was also examined. The mix was in a L$_a$ phase over the investigated range of 0–80°C.

**DISCUSSION**

The X-ray data show dolichol to be a potent mediator for expression of lipid curvature. The fact that dolichol affects the phase behavior of the Ole$_2$-PtdEtn/Ole$_2$-PtdCho mixtures but not of Ole$_2$-PtdCho alone supports the picture of a competition between expression of intrinsic curvature and hydrocarbon packing. For large values of $R_s$, as with Ole$_2$-PtdCho, the L$_a$ phase is preserved upon the addition of small amounts of dolichol. However, when $R_s$ is reduced by mixing in a large amount of Ole$_2$-PtdEtn, the free energy balance shifts toward the expression of curved structures.

At 2% (wt/wt), which is about 1.5 mol %, dolichol promoted the formation of H$_II$ phases in the 3:1 Ole$_2$-PtdEtn/Ole$_2$-PtdCho mixture even at 0°C. This is roughly 50°C below the first onset of H$_II$ formation in the absence of dolichol. Moreover, dolichol promoted a stable balance of coexisting L$_a$ and H$_II$ phases over a 35°C temperature span, a behavior not observed with dodecane (13). Considering the size of the dolichol molecules, one sees that even one molecule represents a high local concentration of additional hydrocarbon volume. Perhaps even a few dolichol molecules, when locally clustered, can promote the expression of local curvature, provided that the intrinsic curvature, $R_s$, is small enough. The protein-mediated clustering of dolichol, in bilayers of sufficiently small values of $R_s$, may be a potent mechanism for the biological control of bilayer stability.

In a larger context, one is led to ask if an organism can afford to let the value of the intrinsic curvature of its membranes vary, given that such variation drastically alters the bilayer stability in the presence of various hydrophobic...
molecules. One suspects not. This leads me to propose that living organisms seek to stabilize the intrinsic curvatures of their bilayers at values peculiar to the membrane concerned. It should be emphasized that this curvature hypothesis is not based on data—to my knowledge, no real biomembrane intrinsic curvature values have been measured yet. My hope in stating the hypothesis is to provide a rationale for future experimentation on the elastic properties of biomembranes. The curvature hypothesis has two parts.

(i) Living cells seek to homeostatically control the net intrinsic curvatures of the bilayer leaflets of their membranes. (ii) Functionally important geometry-dependent local instability of the lipid surface is modulated by local variation of the hydrophobic packing, hydration, and/or electrostatic components of the free energy.

Biomembranes differ from model lipid bilayers in that they contain protein and have an asymmetric bilayer leaflet composition (22). Hence, we suppose that the intrinsic curvatures include the influence of protein and are defined separately for the two monolayers in the bilayer. The second part of the hypothesis is meant to emphasize that if the monolayer intrinsic curvatures are kept constant, then local membrane destabilization must proceed by other mechanisms. An example might be a protein that introduces a long hydrophobic chain into the region to be destabilized. It is of interest to speculate that this might represent a new class of membrane proteins: proteins that have a site-specific part that chooses the location for destabilization and a hydrophobic tail that is dragged along to assist in the destabilization. It is suspected that biomembranes mix bilayer and nonbilayer lipids to adjust \( R_0 \). If so, then the homeostatic control of the intrinsic curvature provides a rationale for the lipid mix present in biomembranes.

In principle, the hypothesis can be tested. As noted earlier, there exist bacteria whose fatty acid synthesis is disabled and, thus, whose membrane lipid composition can be altered by appropriate manipulations of the nutrient medium (3, 4). Imagine a number of such nutrient modifications so as to produce a number of membrane populations. Divide these into healthy and unhealthy classes of bacteria. If stabilization of the intrinsic curvature is important, then we expect that the bacteria in the healthy class will all have similar values of the membrane intrinsic curvature, while the unhealthy class will have divergent values. Although it is not yet clear how the intrinsic curvatures can be measured, a strategy similar to Kirk and Gruner (13) may be possible: lyse the cells to isolate the membranes, pellet the membranes in buffer saturated with butane or pentane, and see if \( H_\Pi \) phases can be induced. If so, the cylinder radii might be taken as an approximate measure of \( R_0 \). The difficulties of this procedure because of, say, membrane asymmetry, charge, or heterogeneity remain to be determined.

If the intrinsic membrane curvature is homeostatically adjusted by cells, then there must exist proteins that are sensitive to the intrinsic curvature. This is not unreasonable, since typical values are <50 Å (10). In addition to mannosyltransferase II (18), the Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum has been shown to be optimally active when reconstituted into bilayers containing a large fraction of nonbilayer lipids (23). In the latter case, many different lipid headgroups were tried, thereby ruling out headgroup specificity.

This suggests that some membrane proteins may require a specific range of intrinsic membrane curvatures in order to function properly. Consequently, the \( R_0 \) specificity of membrane proteins may provide another means of testing the curvature hypothesis. The experimental strategy would, for instance, be to assay protein functional activity when the proteins are reconstituted with various lipids (e.g., see ref. 23). In so far as the protein is dilute in the bilayer, the \( R_0 \) value is nearly that of the pure lipid. The method of Kirk and Gruner (13) may then be used on the pure lipid mix to obtain \( H_\Pi \) phases. The radii of the water cores may be used as a measure related to \( R_0 \). This kind of test may be relatively easy to perform but may be difficult to interpret, since not all proteins are expected to be sensitive to \( R_0 \). Moreover, other lipid factors (e.g., chain length, headgroup type) are probably important to many proteins. Even so, the exploration of the intrinsic lipid-curvature dependence of proteins is a worthy goal since so little is known about membrane proteins.

Note Added in Proof. A recent study of the phase-modulating effect of dolichol (24) has confirmed the behavior of dolichol reported here.

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