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Review

Insolubility of lipids in Triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts)

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Abstract

The insolubility of lipids in detergents is a useful method for probing the structure of biological membranes. Insolubility in detergents like Triton X-100 is observed in lipid bilayers that exist in physical states in which lipid packing is tight. The Triton X-100-insoluble lipid fraction obtained after detergent extraction of eukaryotic cells is composed of detergent-insoluble membranes rich in sphingolipids and cholesterol. These insoluble membranes appear to arise from sphingolipid- and cholesterol-rich membrane domains (rafts) in the tightly packed liquid ordered state. Because the degree of lipid insolubility depends on the stability of lipid-lipid interactions relative to lipid-detergent interactions, the quantitative relationship between rafts and detergent-insoluble membranes is complex, and can depend on lipid composition, detergent and temperature. Nevertheless, when used conservatively detergent insolubility is an invaluable tool for studying cellular rafts and characterizing their composition.

Keywords: Sphingomyelin; Dipalmitoylphosphatidylcholine; Liquid ordered state; Detergent resistant membrane

1. Introduction

The interaction of detergents with membranes was actively explored 25–30 years ago, when the usefulness of detergent solubilization for isolating and characterizing integral membrane proteins was first appreciated [1,2]. In recent years it has become apparent that detergent can solubilize membrane domains in different physical states differentially. This property has provided significant support for the proposal that separate domains with different lipid and protein compositions can exist in cellular membranes [3,4]. These domains are important because recent work has shown that the affinity of certain proteins and lipids for specific membrane domains has important physiological consequences in processes as diverse as cell surface signaling, cell adhesion and motility, and intracellular sorting [5,6]. In this review, we will summarize the effects of lipid physical state upon detergent (specifically Triton X-100)-membrane interactions, and explore the implications of these effects for the domain organization of model and cellular membranes.

2. Physical states of lipid bilayers

2.1. The liquid crystalline and gel states

Lipid bilayers can exist in several physical states

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[7,8]. The 'solid' gel (Lβ) and fluid, liquid crystalline states (Lα, Ld, Lc) are the two most familiar states. In the solid-like gel state lipids are tightly packed, with the acyl chains extended. There is little lateral diffusion. In contrast, in the Lα state the acyl chains are more kinked, packing is loose, and lateral motion is relatively rapid.

2.2. Melting temperature

T_m, the temperature of the gel to Lα melting transition for a pure lipid, is an important parameter in understanding lipid physical state [7,8]. It can be thought of as a measure of the stability of the tightly packed gel state relative to the fluid state. The higher the T_m value, the more stable the tightly packed gel phase. T_m depends strongly on acyl chain structure. Saturated acyl chains impart a high T_m to lipids. T_m also increases with chain length [9]. In contrast, unsaturated acyl chains, which have *cis* double bonds, interfere with tight packing, and they impart a relatively low T_m value to lipids. Sphingolipids (which, as we will see, are likely to be important in domain formation in biological membranes) tend to have relatively saturated chains and thus high T_m values [10,11]. In contrast, natural glycerophospholipids tend to be enriched in mono- or polyunsaturated chains attached to the 2-position of the glycerol, and thus have relatively low T_m. (Some sphingolipids do contain a *trans* double bond in the sphingoid base, but it is in a position close to the bilayer surface. Double bonds at this position do not greatly interfere with tight packing, and lipids containing such double bonds maintain a high T_m [11,12]. Being in the *trans* state further reduces the effect of this double bond. Other sphingolipids have one double bond located far down a very long chain fatty acid, which again would allow facile tight packing [13,14].) Another important factor affecting T_m is polar head group structure. For example, sphingomyelin, which has a phosphocholine attached to the hydrophobic ceramide core, tends to have a lower T_m than glycosphingolipids, which have carbohydrates attached to ceramide [11].

2.3. The liquid ordered state

More recent work suggests that lipids can exist in another physical state that may be of biological significance. This is the liquid ordered state (Lo state, also called γ state in early studies). The Lo state has been identified in some mixtures of lipids and cholesterol in model membranes [15–20]. In the Lo state, acyl chains of lipids are extended and tightly packed. In this sense, the Lo state is similar to the gel state, and lipids that favor gel state formation, and thus have a high T_m in the absence of cholesterol, tend to form the Lo state in the presence of cholesterol [17]. On the other hand, lateral diffusion in the Lo state appears to be almost as rapid as in the fluid Lα state [21,22]. Thus, in a sense the Lo state has properties intermediate between gel and Lα states.

3. Interaction of the detergent Triton X-100 with lipid

3.1. Introduction

The polyoxyethylene detergent Triton X-100 is one of the most widely used non-ionic detergents. When Triton X-100 micelles and lipid vesicles are mixed, they generally form mixed Triton-lipid aggregates. In excess Triton X-100, these aggregates often take the form of mixed detergent/lipid micelles [23,24]. Mixed micelles are much smaller than vesicles, and thus lipid incorporated into mixed micelles is considered to have been solubilized by detergent. However, mixing lipid and detergent does not always lead to solubilization. A mixed bilayer composed of detergent and lipid forms when the detergent/lipid ratio is low [25].

The detergent/lipid ratio is not the only parameter controlling solubilization. The precise way in which Triton X-100 interacts with lipid bilayers also depends strongly upon the physical state of the lipid. Therefore, we must consider how to evaluate solubilization and its dependence on lipid state.

3.2. Assays of detergent solubilization/insolubility

We shall see that the degree of membrane solubilization by Triton X-100 has provided important evidence for the existence of lipid domains in cell membranes. Furthermore, the amount of material insoluble in Triton X-100 is often used to evaluate the degree of lipid domain formation. Therefore, it is

important to discuss how solubilization can be accurately measured. Because of their smaller size, micelles scatter much less light than vesicles. Their small size also allows micelles to be distinguished from vesicles by size chromatography, by the fact that they generally cannot be pelleted by centrifugation, and by their inability to be easily caught on filters [23,24,26]. Micelles are also unlike vesicles in that they do not have an internal aqueous compartment in which substances can be trapped.

The decrease in the light scattering after solubilization of a suspension of vesicles is the simplest way to evaluate solubilization of lipids. The loss of light scattering can be assessed by the optical density (OD) in an absorbance instrument, or intensity in a fluorimeter in which excitation and emission monochrometers are set to equal wavelengths [26,27]. OD depends on the size of the light scattering species, and in ordinary experiments is negligible for micelles relative to that of lipid vesicles. In addition, OD varies in a nearly linear fashion with the concentration of the light scattering species (vesicles), at least over a limited range of concentrations. Therefore, the % loss in OD after detergent addition can reflect % solubilization [26]. This relationship is most accurate when addition of detergent does not change the size of the unsolubilized vesicles. However, this is not always the case. Addition of subsaturating amounts of detergent to a suspension of unilamellar lipid vesicles can increase OD [28–31]. This effect is more pronounced for small unilamellar vesicles (SUV), than for large unilamellar vesicles (LUV), and does not seem to occur with multilamellar vesicles (MLV) [30]. Presumably, the increase in OD reflects both an increase in vesicle size due to the increased number of molecules per vesicle upon detergent addition, and more importantly, vesicle fusion [32]. Another potential ambiguity in interpreting light scattering could arise if detergent addition induced fission of large vesicles into smaller ones [30]. However, to our knowledge this process has not been observed. A final limitation of light scattering measurements is that they do not identify the chemical structure of the insoluble material, and thus cannot be used to detect differential solubilization of lipids in a lipid mixture.

For these reasons, it is important to supplement OD measurements with additional analyses. It is best to quantify the fraction of each membrane component that is insoluble by direct methods such as quantitative HPLC, radioactivity, or phosphate assay [26,33,34]. This requires isolating insoluble material using either centrifugation (pelleting insoluble material or floating it on density gradients), size chromatography, or filtration. These methods for detecting solubilization have their own limitations. Lipid may adhere to the chromatographic media used, and pelleting lipid requires both that the density of lipid exceed that of solution, and that the insoluble species be large enough to pellet. Large enough size for isolation is an issue in filtration methods as well. The trapping of small molecules is probably the most ambiguous method for evaluating resistance to solubilization, as it is not unusual for vesicles to become leaky after exposure to detergent.

3.3. Solubility of lipids in the liquid crystalline state in Triton X-100

Lipids in the Lα state are generally readily solubilized by excess Triton X-100, and form typical mixed micelles [23,24]. In general, the key parameter controlling the degree of solubilization is the ratio of the concentration of detergent in micellar form to lipid [2]. For example, solubilization of egg phosphatidylcholine (egg PC), requires an excess of detergent over lipid. The onset of solubilization occurs at a mole ratio close to 1 Triton/lipid, and the process is complete at 3–4 Triton/lipid [30].

3.4. Insolubility of gel state lipids in Triton X-100

The effect of Triton X-100 on gel state lipids is very different from its effect on fluid state lipids. In the mid 1970s Dennis and colleagues examined the structure of mixed micelles of phospholipids and polyoxyethylene detergents. Ribeiro and Dennis demonstrated in an NMR study that whereas dipalmitoyl PC (DPPC) vesicles are soluble in Triton X-100 in the Lα state (at 49°C), they were detergent insoluble in the gel state (at 20°C) [35]. This was confirmed in subsequent studies by centrifugation and gel filtration chromatography [22,24]. These latter experiments also demonstrated that the fraction of DPPC remaining in vesicular form did not bind much detergent. Thus, the tightly packed gel phase

can be resistant to both detergent binding and solubilization. These observations suggest that Triton X-100 cannot pack tightly against saturated acyl chains, and that as a result, at very low temperatures lipid-lipid interactions between tightly packed acyl chains in the gel state are much stronger than lipid-Triton interactions. Interestingly, however, it was also found that DPPC was soluble in the gel state at 37°C, which is just below the temperature (41°C) at which DPPC forms the Lα state [35]. This behavior is discussed next.

3.5. Solubilization of gel state lipids at very low Triton X-100 concentrations when temperature is close to T_m: a paradox?

Several further studies showed that Triton X-100 could solubilize lipids such as dimyristoyl PC, DPPC and sphingomyelin when they were in the gel state

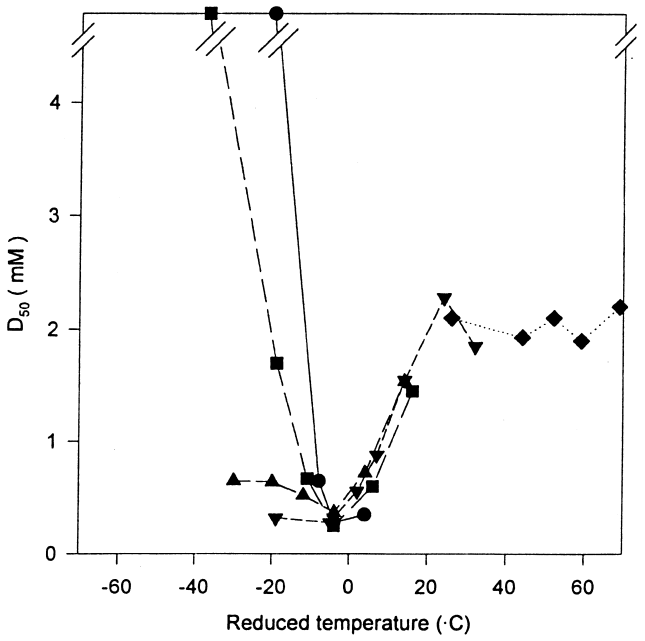

Fig. 1. The temperature dependence of the concentration of detergent required to dissolve lipids. D_{50} is the concentration of Triton X-100 necessary to reduce the optical density of a 1 mM solution of large unilamellar vesicles by 50%. The 'reduced temperature' refers to the difference between the experimental temperature and the melting temperature, T_m. ◆, DOPC; ▼, dimyristoyl PC; ▲, dipentadecanoyl PC; ■, DPPC; ●, distearoyl PC. Egg PC solubilization is similar to dioleoyl PC. Notice the DPPC and distearoyl PC points that are off scale. Adapted with permission from Patra et al. [37].

[31,36–38]. Furthermore, in this state these lipids were fully solubilized at even lower concentrations of detergent than were required to solubilize them when they were in the Lα state (Fig. 1). Likewise, vesicles composed of saturated lipids in the gel state were found to dissolve at lower detergent concentrations than vesicles composed of unsaturated lipids in the Lα state when measured at the same temperature [31,37]. This enhanced solubility of gel state lipids is only found at temperatures relatively close to the T_m of the pure lipid. Thus, it does not contradict the studies at very low temperature, described in the previous section, which examined insolubility of gel phase lipids at temperatures well below T_m.

Nevertheless, it is surprising that while solubilization of gel state lipids is 'impossible' under one set of conditions, it is particularly easy under another. How can such behavior be rationalized? This question can be divided into two. The first is: why can gel state lipids bind detergent when the temperature is close to T_m? This is probably a consequence of the fact that the stability of the gel state is marginal close to T_m. As a result, lipid-lipid interactions may be of comparable energy to lipid-detergent interactions at these temperatures, and the former can be replaced by the latter. Furthermore, the gel state lipids may not be as tightly packed at temperatures close to T_m [39].

The second question is: why should such lipid-detergent mixtures form micelles so much more readily than they do above T_m? One possible explanation involves the well-known dependence of the structures formed by lipids and detergents on their shape. Micelle formation requires molecules to form a 'cone' shape in which the polar head group occupies a space with a larger cross-section than that of the hydrocarbon, whereas in bilayers the polar head group and hydrocarbon chains have similar cross-sectional areas [8]. Thus, the shapes of detergent and lipids in a mixture, as well as their relative concentration, determine whether they form mixed micelles or bilayers. In the gel state, fatty acyl chains have a relatively small cross-sectional area. Maintenance of this type of conformation when lipids are mixed with detergent might aid in micelle formation. It should be noted that the influence of shape on the form taken by detergent/lipid mixtures has already been recognized in other cases [40].

Another, related possibility is that lipid-lipid inter-

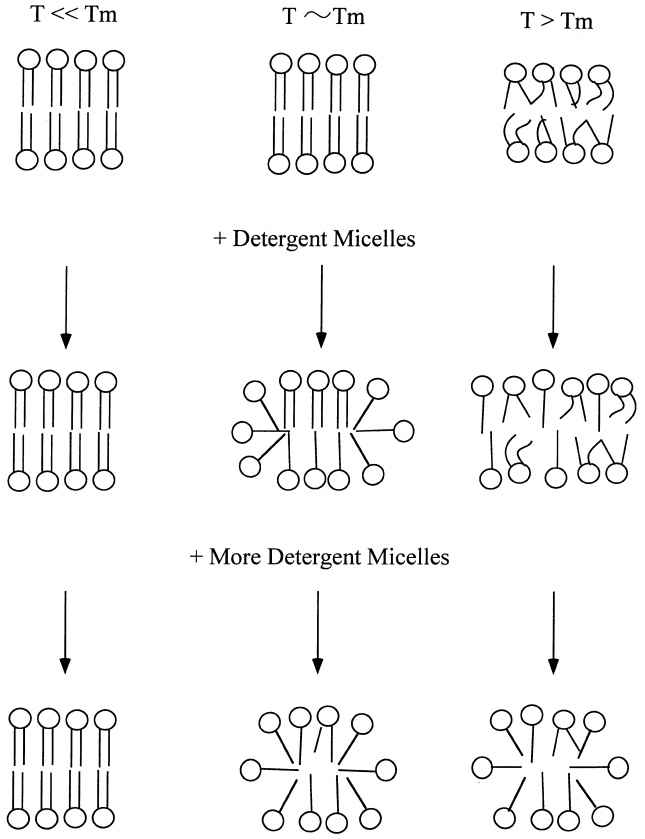

Fig. 2. A schematic illustration of the effect of temperature on solubilization by detergent. At $T \ll T_m$, lipids with long, saturated acyl chains are insoluble, and do not bind detergent well. At T close to T_m, detergents can bind and micelles form at low detergent concentrations due to lipid packing behavior (see text for details). At $T > T_m$ detergents bind well, but form mixed bilayers with lipid at subsaturating concentrations. At higher detergent concentrations micelles form.

actions are strong enough at temperatures close to T_m to allow clusters of lipids to exist within a mixed detergent/lipid aggregate. Such aggregates could form structures with shapes that are favorable for micelle formation if the detergent-rich regions formed the highly curved regions needed for micelles (see schematic illustration in Fig. 2).

3.6. Insolubility of lipids in the Lo state in Triton X-100

Lipids in the Lo state can also be insoluble in Triton X-100. The first hint of this behavior came from early studies in which cholesterol was found to reduce the degree of solubilization of PC by Triton X-100 [36,41]. It was noted that this effect was relatively specific for saturated PC. More recent studies show that the degree of insolubility of such lipid/cholesterol mixtures is closely linked to their physical state. Model membranes consisting of DPPC:cholesterol 2:1, which are in the Lo phase [17], are completely insoluble in Triton X-100 [33,42]. In contrast, model membranes composed of dioleoyl PC (DOPC)/cholesterol 2:1, which appear to be in the Lα phase as judged by fluorescence polarization and quenching [27,33], are fully soluble in Triton X-100.

4. The behavior of mixtures of low-T_m and high-T_m lipids: domain formation

4.1. Lipid mixtures can form coexisting gel and liquid crystalline domains

It has long been known that model membranes composed of mixtures of high-T_m and low-T_m lipids contain a mixture of coexisting Lα and gel domains over a range of temperatures. Many studies have shown that the behavior of such mixtures can be described by standard phase diagrams that give both the amount of lipid present in each type of domain and the lipid composition of each type of domain at any temperature. The reader is referred elsewhere for more information on this subject [9,43].

4.2. Evidence for coexisting Lo and Lα domains: cholesterol can promote domain formation

The question of whether Lo and Lα domains can coexist has only been investigated relatively recently. A number of phase diagrams defining a region of coexisting Lo and Lα domains have been proposed for the binary mixtures of DPPC and cholesterol, but they differ significantly among themselves with regard to the exact conditions under which domain coexistence would occur [17–19,44]. This disagreement is probably related to the similarity of physical properties in the Lo and Lα state, which makes it difficult to differentiate between them.

Stronger evidence for the coexistence of Lo and Lα domains comes from studies in model membrane vesicles containing mixtures of a high-T_m lipid, a low-T_m phospholipid and cholesterol. Such mixtures

are a reasonable (although crude) model of cholesterol-containing cell membranes like the plasma membrane. Domain formation in such mixtures has been investigated by a fluorescence quenching approach [45]. This method involves inserting a fluorescent probe into a lipid mixture that includes a lipid carrying a quencher of fluorescence. As a result fluorescence intensity is sensitive to the presence of quencher lipid-rich and quencher lipid-poor domains, largely irrespective of their other physical properties.

Using this approach, recent studies have elucidated two important aspects of the influence of cholesterol on domain formation. The first is that Lo and Lα domains can coexist and the second is that cholesterol can promote lipid phase separation resulting in the formation of coexisting domains within a previously uniform bilayer.

Silvius and colleagues constructed a tentative three component phase diagram describing the lipid states present in mixtures of DPPC, a low-T_m analog of a short chain PC with a quencher attached, cholesterol, and a small amount of fluorescent probe [20]. Increasing cholesterol (up to about 30%) promoted formation of Lo domains. That is, separate Lo and Lα phase domains were present in bilayers containing concentrations of DPPC too low to undergo phase separation and the formation of gel phase domains in the absence of cholesterol. However, at very high cholesterol concentration there was some evidence of a decreased tendency to form coexisting domains. Instead, these mixtures seemed to form a uniform Lo phase.

In a second study [27], we examined the coexistence of Lo and Lα domains in mixtures of DPPC or sphingolipids and a low-T_m, quencher-bearing PC different from the one used in the Silvius study. Fluorescence quenching with and without 33 mol% cholesterol was examined in each case. As in the Silvius study, cholesterol promoted formation of coexisting Lo and Lα domains. Interestingly, as the sphingolipid concentration was increased, Lo domains first appeared at a sphingolipid concentration of 20 mol%. This is well within the level expected to be present in the outer leaflet of the plasma membrane under physiological conditions, and suggests that Lo domain formation is likely to occur in natural membranes.

4.3. How does cholesterol promote domain dormation?

Cholesterol is believed to interact particularly tightly with saturated acyl chains [13,46]. The relationship between tight saturated lipid/cholesterol packing and domain formation was examined in a fluorescence quenching study comparing domain formation in mixtures of a low-T_m quencher-bearing PC, the high-T_m saturated lipid DPPC, and a series of sterols with differing tendencies to promote tight lipid packing [26]. A close correlation was found between the ability of a sterol to support domain formation and, as measured by a variety of parameters, its ability to pack tightly with DPPC [26,47,48]. Some sterols were even found to antagonize tight packing and domain formation. These experiments suggested a model in which cholesterol acts as a 'glue' promoting self-association of high-T_m lipid in a tightly packed detergent-resistant cluster that also contains sterol.

It should be noted that the conditions of this study may limit its relevance to cellular membranes. One caveat is that to optimize the ability to detect domains these experiments were only done at 15 mol% sterol. The conclusions would be more definitive if similar behavior were observed at more physiologically relevant high cholesterol concentrations. In addition, it is not known whether sphingolipid-containing mixtures will show behavior similar to that of DPPC. However, a similar correlation between sterol structure and insolubility in detergent has been observed for a few sphingomyelin/sterol mixtures [49].

5. Detergent insolubility of cellular membranes and its relationship to Lo state sphingolipid/cholesterol domains (rafts)

5.1. Partial insolubility in detergent: evidence for lipid domains?

The observation that mixtures of low- and high-T_m lipids can form separate gel and Lα domains gave rise many years ago to the proposal that similar lipid domains might exist in biological membranes. One possibility was that gel state sphingolipid do-

mains might coexist with predominating fluid glycerophospholipid domains in the plasma membrane of eukaryotic cells [50]. Early detergent insolubility studies provided some evidence in support of this model. As the cytoskeleton is detergent insoluble, most early work on the detergent insolubility of cell membrane proteins and lipids was interpreted in terms of cytoskeletal attachment. However, a relationship between sphingolipid clustering and detergent insolubility was suggested by Yu et al. in 1973 [51]. Using erythrocytes, these investigators isolated detergent-insoluble sphingolipid-enriched membrane fragments from erythrocytes. Although they were associated with the cytoskeleton, it was speculated that these insoluble membranes might originate from sphingolipid clusters present in the tight erythrocyte membrane prior to detergent treatment. Several subsequent studies identified and characterized the composition of similar detergent-insoluble lipid fractions from a variety of cell types and platelets [52–59].

5.2. *Problems with the possibility of gel-like domains in cells*

Despite suggestive results from these and many other studies, several considerations argued against the possibility of coexisting lipid domains in cellular membranes. One problem was the difficulty of visualizing such domains [44], especially in cells. In addition, the presence of high levels of cholesterol in cells seemed problematic. Cholesterol was known to abolish the transition between the gel and Lα states in pure phospholipids, and to induce properties intermediate between those of the gel and Lα states [44,60]. Thus, it could be presumed that cholesterol would prevent the separation of a membrane into distinct gel and fluid state lipid domains. A final objection to the idea of gel-like domains was that the gel state did not seem to be suitable to support biological functions. Gel domains tend to strongly exclude proteins [61,62], and lateral diffusion within such domains is very slow [21]. Furthermore, bilayers that contain both gel and Lα domains tend to be permeable to small molecules. How could dynamic cellular processes be maintained in such an environment?

5.3. *Additional evidence for lipid domain formation and function in cells*

The possibility of a cellular function for sphingolipid domains helped keep the domain proposal alive. Van Meer and Simons proposed that glycosphingolipid-rich domains (rafts) might be involved in lipid and protein sorting to the glycosphingolipid-rich apical membrane of epithelial cells [63]. Matlin and colleagues then showed that influenza hemagglutinin became detergent insoluble during biosynthetic transport to the apical membrane of epithelial cells [64]. It was suggested that this might occur through association with sphingolipid domains of the type observed by Yu et al. [51], and that this association might be important in protein transport and possibly in sorting in the late secretory pathway.

Isolation of a Triton X-100-insoluble membrane fraction from epithelial cells provided further support for the idea of lipid rafts [65]. These membranes were enriched in glycosphingolipids and glycosylphosphatidylinositol (GPI)-anchored proteins, both of which are apically targeted. Thus, this study was consistent with the raft model for sorting, and suggested that rafts could be isolated by their detergent insolubility. More detailed characterization showed that these membranes were also enriched in both sphingomyelin and cholesterol. Sphingomyelin is generally not enriched in the apical membranes of epithelial cells. Because of this and other factors, the exact role of these membranes (or more precisely, of the membrane domains from which they are believed to arise) in epithelial cell sorting has not yet been fully defined [6]. However, excitement about the structure and function of these domains had been ignited. More recent studies have suggested additional roles for these domains in a variety of biological processes, especially signal transduction at the cell surface [5,6,66,67].

It should be noted that Triton X-100 is not the only detergent in which insolubility of rafts or sphingolipid/cholesterol model membranes can be observed [65,67–69]. Of particular physiological interest is the possibility that insolubility of raft lipids in natural bile salt detergents can explain the phospholipid composition of bile [67].

To avoid confusion, we should define the distinc-

tion between membranes that can be isolated from detergent lysates, and domains that may exist in cells. Detergent-resistant membranes (DRM) will refer to the detergent-insoluble, sphingolipid/cholesterol-rich membranes obtained when cell membranes are treated with Triton X-100. This material has also been termed DIG (detergent-insoluble glycolipid), TIFF (Triton-insoluble floating fraction), and GEM (glycolipid enriched membranes). The term raft will be restricted to the sphingolipid/cholesterol-rich domains believed to exist in cell membranes prior to detergent treatment. This distinction is important because the detergent-insoluble membranes and rafts are not necessarily identical, as is discussed in Section 6.

5.4. The liquid ordered state (Lo) raft hypothesis

Determination of the lipid composition of these Triton-insoluble membranes provided an important foundation for further studies that established the current model for the structure of what are now called sphingolipid/cholesterol rafts. The insolubility of cellular membranes could be mimicked in model membranes composed of sphingolipids, phospholipids and cholesterol [33]. This result suggested that detergent insolubility of DRMs did not require an underlying protein framework, as had been supposed in previous work on detergent insolubility of cell membranes. Instead, detergent insolubility was an inherent property of the lipids.

Studies on a variety of model membranes were undertaken to characterize the lipid compositions required for insolubility [33]. At the time, the insolubility of ordered state lipids was not widely appreciated, but it became clear that the lipid compositions supporting insolubility were the same as those that would form the Lo state (and the gel state). It was found that model membranes known to be in the Lo state were detergent insoluble. In addition, the physical state of detergent-treated model membranes mimicking DRM matched that of Lo state material, as measured by fluorescence polarization. Together, these findings led to the idea that the partial detergent insolubility of cell membranes might reflect the presence of domains in different physical states [33]. In particular, the high concentration of sphingolipids and cholesterol in DRMs suggested that detergent-insoluble rafts in an Lo-like state might coexist with detergent-soluble Lα state domains in cell membranes.

Several additional lines of evidence now support the Lo raft model. The studies described in Section 4.2 show that coexistence of Lo and Lα state domains in membranes with a lipid composition similar to that of the plasma membrane is plausible. In addition, the physical properties of DRMs isolated from mammalian cells are very similar to those of genuine Lo state model membranes [70,71]. Finally, a series of experiments with model membranes demonstrated that the effect of cholesterol depletion on detergent insolubility of GPI-anchored proteins in cells and in Lo state model membranes could be explained by the ability of cholesterol to promote formation of the detergent-insoluble Lo state [42].

The presence of certain proteins in DRMs also suggests that DRM lipids are present in an ordered, Lo-like state. In particular, proteins that are modified with saturated lipid moieties would pack well into Lo state rafts. Such proteins include GPI-anchored proteins (which contain predominantly saturated acyl chains) and proteins linked directly to saturated acyl chains, such as Src family protein tyrosine kinases, and heterotrimeric G protein α subunits [5]. All these are enriched in DRMs. Purified GPI-anchored proteins [33,42] and G-α subunits [72] are also enriched in detergent-resistant membranes derived from model membranes of appropriate lipid composition [5]. In contrast, transmembrane polypeptides, which would not be expected to pack well into an ordered lipid environment, are generally (with several interesting exceptions that will require further characterization) depleted from DRMs [5].

Finally, the recent introduction of detergent-independent methods for visualizing domains (especially after clustering of putative domain components) and of studying their function provides complementary support for this model [5,73,77].

In summary, the idea that Lo phase rafts exist in biological membranes is well supported by model membrane studies. This model also explains the behavior of DRMs isolated from cells, and why certain proteins and lipids are enriched in them. Detergent insolubility has proven to be a useful and important tool for characterizing these domains.

It should be noted that the Lo raft model contrasts

with an earlier proposal that the entire plasma membrane might be in the Lo state [74]. However, the exact phase behavior of lipid mixtures is so poorly understood that a model in which plasma membranes sometimes exist in a uniform state that is intermediate between Lα and Lo states, including an intermediate state close to an Lo state, cannot be ruled out at this time [4].

5.5. *Rafts in the cytoplasm-facing leaflet of the lipid bilayer: head group structure and its effect on raft formation*

The Lo raft model can explain lipid domain formation in the sphingolipid-rich outer leaflet of the plasma membrane. But what happens in the inner leaflet of the plasma membrane, which is believed to be relatively sphingolipid-poor? One possibility is that rafts only form in the outer leaflet of the membrane. However, DRM have a bilayer-like appearance. In addition, acyl chain-anchored proteins, which bind to the inner leaflet of plasma membranes, often associate with DRM [5,75,76]. This association can be important for their function [75]. Furthermore, recent studies showing that plasma membrane glycerophospholipids have more saturated chains than those in other membranes also hint that glycerophospholipids might support raft formation in the inner leaflet [78,79]. Finally, ordered domains in outer leaflet may promote ordered domain formation in the inner leaflet [80,81].

Two studies show that a long, saturated acyl chain structure can be more important for determining the association of a lipid with Lo domains than the specific structure of its polar head group [33,71]. Nevertheless, because head group structure can strongly modulate lipid packing, the polar head group structures of glycerophospholipids in the inner leaflet may contribute to raft formation. For example, phosphatidylethanolamine (PE) shows substantially higher T_m values than a PC molecule with a identical acyl chain structure [82,83], and is present in high concentrations in the inner leaflet, where it may play a role similar to that of sphingolipids in the outer leaflet. An alternate possibility is that other high T_m lipids, such as ceramide, may be important for raft formation in inner leaflets. Of course, this is all simply speculation at present.

6. Quantitative aspects of evaluating raft formation from insolubility

6.1. *There is a correlation between the extent of Lo raft/lipid domain formation and the extent of insolubility*

A persistent concern in using solubilization to evaluate domain formation is how the addition of detergent perturbs the domain structure of membranes [51]. How closely does the amount of insoluble material obtained reflect the amount of lipid in domains prior to detergent addition? Is it possible for detergent treatment to create ordered domains by selective extraction of low-T_m lipids from a uniform bilayer? Recent improvements in the ability to detect the extent of Lo raft formation by methods other than detergent insolubility, described above, allow investigation of these questions. One example is the study described in Section 4.2 in which the amount of Lo domain formation as a function of increasing sphingomyelin concentration was measured in a sphingomyelin/PC/cholesterol mixture using fluorescence quenching [27]. The sphingomyelin concentration at which Lo domains first appeared was the same at which detergent insolubility was first detected. In addition, the sphingomyelin concentration at which maximum insolubility was observed appeared to coincide with that at which the entire bilayer was in the Lo state [27]. In a second study, the insolubility of a DPPC/cholesterol mixture as a function of cholesterol concentration was measured [42]. The cholesterol concentration at which insolubility first appeared, and that at which full insolubility was observed, also corresponded well to those predicted from a spectroscopically determined phase diagram [17]. (These data are less definitive because of the uncertainty concerning the exact phase diagram for DPPC/cholesterol.) A third set of experiments involved model membranes containing any of several sterols and sterol analogues [26]. The relative order of sterol promotion of insolubility showed a strong correlation with the relative degree of ordered domain formation in the presence of sterol. In all of these examples, insoluble lipid was only recovered from samples known to contain ordered domains from independent measurements. This suggests that insoluble lipid arises from pre-existing ordered lipid

domains, and is not an artifact of the selective extraction of low-T_m lipids by detergent.

6.2. How insolubility can underestimate or overestimate raft formation

Despite the results described above, the amount of insoluble lipid recovered after addition of detergent to a two phase lipid mixture is unlikely always to be an accurate measure of the amount in Lo domains prior to detergent addition. For example, in the experiments on sterol analogues (described above) the fraction of lipid that was insoluble was in some cases less than the amount of lipid in ordered domains [26]. Such results should be observed whenever detergent has a finite ability to dissolve lipid in an ordered state. In fact, as temperatures approach T_m the gel state is progressively less stable, and we have seen that under these conditions solubilization can be complete even when all the lipid was in the gel state prior to detergent addition. The ability of cholesterol to stabilize tight packing should ameliorate this problem, but the degree to which detergent insolubility underestimates raft formation is impossible to predict at present.

This behavior has important implications for the interpretation of detergent insolubility in cells. Solubilization of cell membranes at 37°C yields little pelletable detergent-insoluble material. In order to obtain DRM from cells detergent extraction is generally performed at 4°C or below. This might mean that rafts only exist at low temperatures. However, as detergent-free methods have also provided support for the existence and functional importance of rafts in cells, it is more likely that whatever rafts exist at 37°C are not stable enough to resist detergent. If this is correct, low temperature allows rafts to be isolated by stabilizing their lipid-lipid interactions and thereby enhancing their insolubility in detergent. Unfortunately, cooling membranes that contain both fluid and ordered domains increases the total amount of lipid in the ordered phase. In fact, at lower temperatures even lipids with a low level of unsaturation may form the Lo state [74,84], and some insolubility of natural PCs can be detected [42].

Thus, performing solubilization at low temperature can lead to an overestimation of the amount of material that is in rafts at the physiological temperature (Fig. 3). This problem may explain why a surprisingly high fraction of plasma membrane lipids can be detergent insoluble, and why the composition of DRM can be so close to that of the plasma membrane as a whole [70,85].

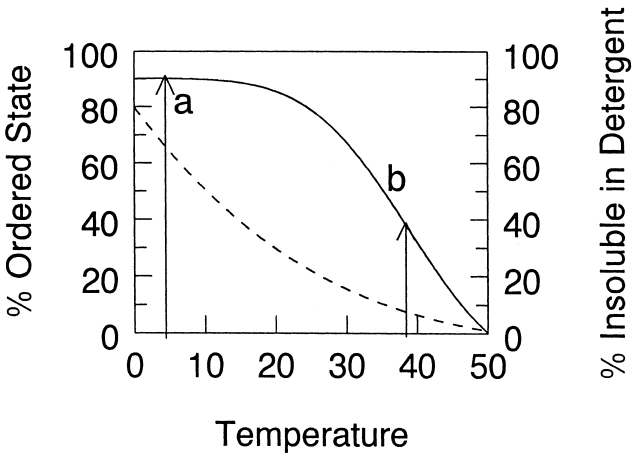

Fig. 3. Schematic illustration of the effect of temperature on % ordered state and % insolubility for a hypothetical lipid mixture containing low- and high-T_m lipids. At all temperatures, % insolubility (- - -) lags behind the % ordered lipid (———). At physiological temperature (b) ordered state lipid is present, but insolubility is almost negligible. At the experimental solubilization temperature (a) insolubility can be detected, but the amount of ordered lipid domains may be greater than at physiological temperature (b) (and in addition their composition could be altered).

The fact that phase diagrams for lipid mixtures containing cholesterol suggest that under some conditions a homogeneous state can exist with properties intermediate between those of the Lo and Lα states [16,17,20] suggests another possible ambiguity in interpreting detergent insolubility data. A homogeneous intermediate state might exhibit partial detergent insolubility, and allow differential solubilization of different lipid components. This could lead to a serious misinterpretation if mistaken for the behavior of a state in which coexisting Lo and Lα state domains exist.

6.3. Does detergent solubilization reach an equilibrium?

An important issue is whether solubilization reaches an equilibrium. Kinetics experiments under conditions of partial solubilization show a plateau in the level of solubilization vs. time after detergent

addition, consistent with (but not proving) the proposal that an equilibrium is achieved (see Fig. 4 and [86]). Another observation consistent with the idea that solubilization reaches equilibrium involves temperature shifts. In some cases in which the lipid solubilization is enhanced by increasing temperature, reformation of a species large enough to scatter light is observed upon cooling ([24] and our unpublished observations). This suggests that solubilization can spontaneously reverse when conditions are changed such that the fully solubilized state is no longer the equilibrium state.

However, other experiments indicate that detergent-lipid mixtures do not always achieve an equilibrium state. This is demonstrated most clearly by experiments using molecules with a high affinity for Lo domains. When these are initially incorporated into vesicles containing a mixture of Lo and Lα domains, they are found in association with the Triton X-100-insoluble fraction after detergent extraction. However, if they are first incorporated into vesicles in the Lα state, and then mixed with vesicles containing both Lo and Lα domains prior to detergent addition, then they are fully solubilized by detergent [42]. Thus, it appears that after solubilization molecules become trapped and cannot freely exchange between DRM and micelles.

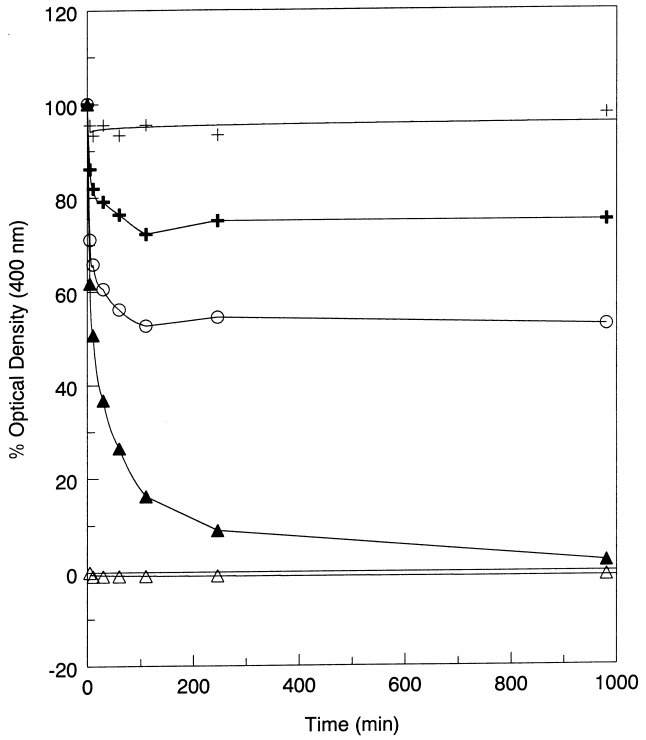

Fig. 4. Kinetics of Triton X-100-induced solubilization of multilamellar vesicles composed of (unless otherwise noted) phospholipid/33 mol% cholesterol mixtures. ▲, DPPC (no cholesterol); +, DPPC/cholesterol; △, DOPC/cholesterol; ○, DPPC/DOPC (1:1) with cholesterol; +, DPPC/DOPC (4:1) with cholesterol. Total lipid concentration was 0.95 mM and Triton X-100 concentration was 0.5% (w/v). Experiments were performed at room temperature in 10 mM phosphate, 150 mM NaCl.

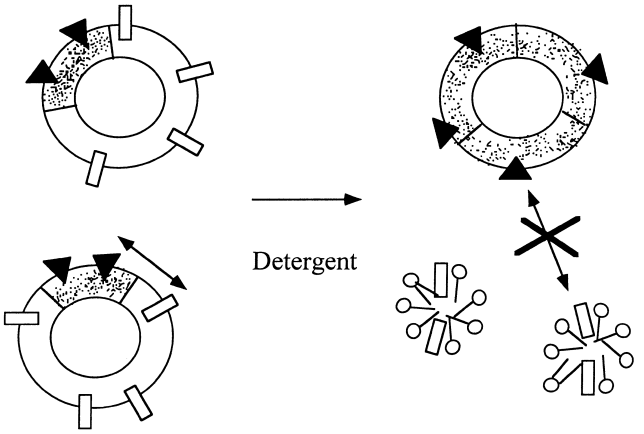

Fig. 5. Schematic illustration of 'trapping' subsequent to the addition of detergent. Shaded regions are bilayer domains in the Lo state. Unshaded regions are in the Lα state. Triangles and rectangles represent components that preferentially associate with Lo and Lα domains, respectively. Prior to solubilization, components can exchange between coexisting domains by lateral diffusion. After solubilization, exchange cannot occur because it would involve passage of hydrophobic molecules through the aqueous solution.

A possible mechanism for how molecules become trapped in DRM is shown in the simple model illustrated in Fig. 5. It suggests that if rafts form closed DRM vesicles upon membrane solubilization, then exchange might require transfer through aqueous solution. Such transfer of extremely hydrophobic species should not occur on any reasonable time scale.

6.4. *Detergent insolubility may result in a kinetically trapped equilibrium*

An important question is whether the distribution of molecules between Lo and Lα domains prior to detergent addition is the same as the distribution between detergent-insoluble membranes and micelles after detergent addition. The degree to which mole-

cules can move into and out of Lo state domains is an important consideration in trying to answer this question. Prior to detergent treatment, individual molecules should rapidly exchange between the Lo domains and the remainder of the bilayer. Such behavior has been observed in intact membranes by single molecule tracking [87]. This contrasts with the above-described lack of exchange of molecules between Lo state vesicles and micelles after solubilization.

Ironically, the fact that molecules cannot exchange between micelles and vesicles may allow detergent insolubility to yield a more accurate evaluation of the equilibrium distribution of molecules between Lo state rafts and the remainder of the bilayer than it would in a situation in which such exchange does occur. If only those molecules that are located in the Lα state domains in a membrane are solubilized, and if they are solubilized efficiently, then in the absence of exchange the distribution of a molecule between insoluble and solubilized fractions will reflect the equilibrium distribution in Lo and Lα domains prior to detergent addition. If, instead, exchange can occur after solubilization, then the distribution of molecules between Lo state vesicles and micelles could be quite different than that between Lo domains and Lα domains prior to detergent addition. In this case, both the affinity of a molecule for micelles and the concentration of micelles would influence the vesicle/micelle distribution.

Despite the inability of molecules to exchange between vesicles and micelles, we cannot rule out the possibility that molecules can redistribute to some degree between Lo domains and whatever mixed detergent/Lα lipid species forms transiently during the solubilization process. In this case, the final distribution between solubilized and insoluble material may not exactly reflect the distribution between Lo and Lα domains prior to detergent addition. Furthermore, whether trapping would occur when the starting material is a homogeneous membrane with properties intermediate between the Lo and Lα states, is not known.

Another consequence of this model is that upon solubilization small rafts are likely to coalesce into larger ones (Fig. 5). As a result, raft morphology in cells cannot be inferred in any detail from DRM morphology. This is important to note because the actual size of rafts in cells is controversial. Different methods give sizes that range from minirafts of perhaps a hundred or so molecules to rafts large enough to detect easily by light microscopy. In fact, in vivo raft size may be controlled by the physiological state of a cell [4,5,88].

7. Conclusion

Tightly packed lipids are able to resist solubilization by detergent under some conditions. The isolation of insoluble membrane fractions has been a key factor in our ability to understand the origin and structure of tightly packed sphingolipid/cholesterol-rich membrane rafts. Furthermore, insolubility can yield information both about raft composition and about how rafts are affected by physiological processes. However, the limitations of this approach must be clearly understood to avoid experimental artifacts. As a result, future progress will depend increasingly on the development of detergent-independent methods for the analysis of membrane rafts.

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