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The fragmented state of lipid bilayers in water: Discovery of a lower consolute point

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Abstract. The bilayers of some typical biological membrane lipids such as PC and DGDG disintegrate in a large excess of water to form an optically invisible dispersive bilayer phase. 'Dark bodies' can be reversibly precipitated from it by raising the temperature. The dispersive phase probably consists of 'knotted sticks', i.e. very thin nodular tubes of bilayer.

After reviewing pertinent experimental and theoretical work we report on the discovery of a lower consolute point near room temperature in DGDG/water systems. Its existence shows that the dispersive phase and the dark bodies belong to the same fragmented (or nodular) bilayer state, representing its expanded and condensed phases, respectively, above the critical temperature.

Keywords. Fragmented state; lipid bilayers; lower consolute point.

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1. Introduction

The fluid bilayers of typical biological lipids and their vesicles are well-known model systems of biological membranes and cells. Among the most common examples are phospholipids such as phosphatidylcholines (PC) and glycolipids such as digalactosyldiacylglycerols (DGDG). Lipid bilayers can assume several phases at physiological temperatures. This may be biologically relevant, and it is physically amazing because only one or two of them can be thermodynamically stable. Therefore, the phases and their transformations are an attractive subject for physical studies.

Our contribution to these Proceedings starts with a summary of the phases and their interpretation in terms of bilayer bending energies. A phase is called regular or anomalous, depending on whether Hookean bending elasticity is enough or higher-order terms in the principal curvatures are needed in addition to explain it. The emphasis is on the anomalous phases. After this preparation we will present new light-microscopic observations made on highly swollen DGDG/water systems. They reveal, among other things, a lower consolute point of an anomalous bilayer state which we name fragmented.

2. Known lipid bilayer phases

There are several regular phases of fluid lipid bilayers in water. They may be divided into at least three classes: First, planar phases such as multilayer systems with various membrane spacings. Second, bicontinuous cubic phases in which a multiply self-connected bilayer

separates two interpenetrating aqueous systems. Third, vesicular phases of generally small vesicles in thermodynamic equilibrium.

Nonplanar multilayer systems forming arrays of either confocal domains or onions may be regarded as additional phases, but thermodynamic stability (or metastability) is particularly difficult to achieve and specify in these cases.

The bilayer bending elasticity in its usual, Hookean form largely determines the free energies of these phases relative to the single flat membrane. In the Hookean case, the energy surface density g comprises only the terms that are quadratic or linear in the principal curvatures c_1 and c_2 of the membrane. A standard formula is

$$g = \tfrac{1}{2}\kappa(c_1 + c_2)^2 - \kappa c_0(c_1 + c_2) + \bar{\kappa}c_1c_2, \tag{1}$$

where κ is the bending rigidity and $\bar{\kappa}$ the elastic modulus of Gaussian curvature c_1c_2. The linear term containing the spontaneous curvature c_0 enters only if the bilayer is asymmetric. Therefore, it vanishes in thermodynamic equilibrium.

In dealing with regular phases, terms of higher than quadratic order in the principal curvatures have been occasionally invoked, but only to explain why sonicated vesicles and the lattice parameters of the cubic phases are of finite size. The situation seems to be totally different in the case of the anomalous lipid bilayer phases, first suspected on indirect evidence [1] and then found by means of electron microscopy. They appear to have in common very strong curvatures, their radii being of the order of the bilayer thickness of 4 nm, and a preference for saddles, i.e. negative Gaussian curvature. If they are to be interpreted in terms of bending energies it is necessary to go beyond Hookean elasticity.

The first anomalies were detected in vesicle membranes by cryo-transmission electron microscopy [2]. The PC bilayers used in those studies occasionally displayed isolated sharp bends. More typical was a graininess (grain size 4–6 nm) covering the whole surface of a vesicle, thus probably being a thermodynamically stable or metastable state. These anomalies do not destroy the basic planarity of the membrane or, in other words, do not change its topology.

Another type of anomaly was found in freeze-fracture electron microscopy of PC vesicles [3]. Some of the vesicle dispersions examined had more or less completely transformed into a fuzz of separate 'knotted sticks' spread over the whole field of view. The knotted sticks seem to represent a dispersive phase of lipid bilayers. The existence of such a phase had previously been inferred from the reversible generation of 'dark bodies' in suitably prepared, highly swollen PC/water system [4].

The structure of the knotted sticks is difficult to analyse because of artifacts of replica formation. However, the micrographs strongly suggest that they are rather stiff chains of minute bilayer spheroids, with diameters down to 20 nm, connected by very narrow bilayer necks (or passages). A small fraction of the sticks were branched, preferably with 120° angles. Figure 1 shows idealized drawings of a knotted stick and a branching. The shapes seen in the micrographs are not as regular.

The graininess and the nature of the knotted sticks have both been explained in terms of a bending energy surface density extended by three higher-order terms,

$$g = \tfrac{1}{2}\kappa(c_1 + c_2)^2 + \bar{\kappa}c_1c_2 + \kappa'[\vec{\nabla}(c_1 + c_2)]^2 + \bar{\kappa}_2(c_1c_2)^2 + \bar{\kappa}_4(c_1c_2)^4. \tag{2}$$

The first two additional terms are of fourth order in the curvatures, while the last is of the eighth. The typical magnitude of the bending rigidity, $\kappa \approx 10^{-19}$ J, is known from

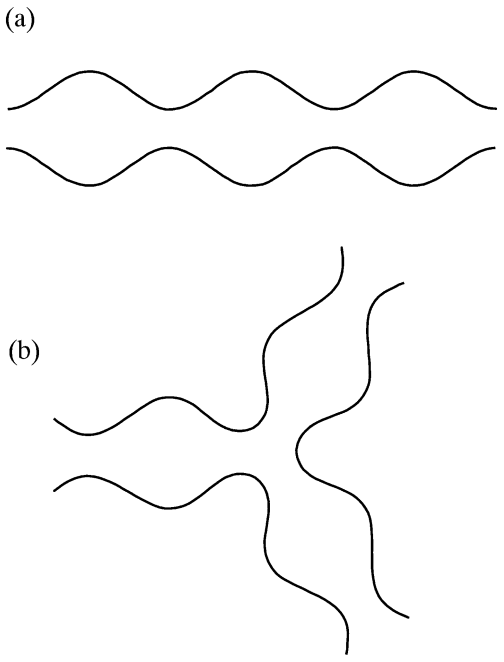

Figure 1. Schematic contours of a knotted stick (**a**) and a branching (**b**). The knot diameter is roughly 25 nm.

experiment. The modulus of Gaussian curvature, $\bar{\kappa}$, enters only in changes of topology because of the Gauss–Bonnet theorem. There are many reasons to believe that it is positive for electrically neutral lipids such as PC and DGDG. The elastic moduli of the gradient term and the $(c_1 c_2)^2$ term have been estimated to be $\kappa' \approx 3 \cdot 10^{-38}\,\mathrm{Jm}^2$ [5] and $\bar{\kappa}_2 \approx -1 \cdot 10^{-36}\,\mathrm{Jm}^2$ [6] on the basis of particular monolayer models. A crude value for the eighth-order modulus, $\bar{\kappa}_4 \approx 1 \cdot 10^{-71}\mathrm{Jm}^6$, was obtained by dimensional analysis from the model underlying the estimate of $\bar{\kappa}_2$. Note that the $(c_1 c_2)^2$ term generally lowers the bending energy density of the membrane because of the negative sign of $\bar{\kappa}_2$. This term is responsible for the transformation of the flat state into one of the 'superstructures' giving rise to the anomalous phases. An energy barrier has to be overcome in each of these transitions. Numerous other terms of even orders up to the eighth are missing in eq. (2), e.g. three more fourth-order terms. We adopted the simple ansatz because the calculation of the remaining moduli, especially those of sixth and eighth order whose number is unknown, appears rather forbidding.

So far, the model based on the five-term energy density (2) has been surprisingly successful. Using values for the elastic moduli of the orders of magnitude given above, we obtain in Monte Carlo simulation an egg carton pattern [6, 7] and, as its molten state, the grainy superstructure [7] of the basically planar membrane. Without any detailed calculation it is obvious that the model can also explain the knotted sticks and their branchings. The main factors here are the highly constricted connections between the knots. This is because of their very strong saddle curvature which contributes only

to the negative $\bar{\kappa}_2$ term. The same term will lower the bending energy of the spheroids, but to a lesser extent because of weaker principal curvatures. The latter effect is obviously not strong enough to result in spontaneous vesiculation.

3. New observations

3.1 *Introductory remarks*

Let us now present some new light-microscopic observations made in a study of highly swollen DGDG/water systems. DGDG, unlike PC, requires the presence of salt (in low concentration) to form dark bodies. In addition to the dark bodies we found the following new phenomena.

To begin with, most of the dark bodies developed a bright border and lost their darkness when they exceeded a certain size. This change in appearance of bodies of increased lipid density is a consequence of phase contrast. In very big bodies we noted for the first time 'bright bubbles' of diminished lipid density. They also changed their appearance with increasing size. To avoid confusion, we will henceforth refer to the enclosed phase as condensed or expanded, depending on the lipid concentration relative to the outside, rather than to the dark or bright appearance of bodies and bubbles.

The bodies of condensed phase and bubbles of expanded phase could both be reversibly produced in the other phase by raising the temperature. The coexistence region of the two phases was found to be limited by a lower consolute point at $T_c \approx 24°C$. This critical point was difficult to detect because, as a rule, 'mature' bodies of condensed phase are not visibly affected when brought to and kept at lower temperatures.

The two coexisting phases and the single phase below T_c probably all consist of knotted sticks, either dispersed or aggregated by branchings. Together, they represent a bilayer state which we may tentatively call fragmented or nodular.

Finally, we will briefly report on 'voracious bodies'. These are bodies of condensed phase caught in the process of sucking up other bilayer material arriving in the form of cylinders or 'ropes'.

3.2 *Experimental*

DGDG extracted from either spinach or comfrey was purchased from Lipid Products (Redhill, Surrey, UK), the indicated purity being 99%. A quantity of the chloroform–methanol solution delivered was transferred with a micropipette onto an object slide and left overnight under vacuum to evaporate the solvent. After covering the residue with a drop of 5–20 mM NaCl solution in distilled and degassed water, the cover slip was put on top and the sample cell sealed with paraffin melting at 65°C. The cells enclosed little or no air and their diameter and height were about 1 cm and 50–80 μm, respectively. The calculated lipid concentration varied from 0.5% to 1% wt./wt.

The samples were swelled for 2 to 6 days, part of the time at elevated temperatures of typically 50°C, before the phenomena shown in the video photographs below took place.

Observation was by video under an optical microscope (Leitz, Ortholux II PolBK) usually in phase contrast. The polarization mode was used to check for birefringence.

3.3 *Some general observations*

Swelling started within a few hours after sample preparation, producing many different structures. They included myelin fingers and paucilamellar cylindrical vesicles frequently attached to the dot of deposited lipid. After at least a day dark bodies appeared, growing either in a highly swollen multilayer system by thinning it further (so that sometimes single membranes became discernible) or next to a multilayer system that gave off lipid material presumably in the form of dispersive phase.

When the dark bodies reached a certain size they often turned bright. This process started in the middle (by contrast reversal) and just outside the circumference (as a halo effect). Above a diameter of ca. 20 μm such a body of condensed phase looked bright in its outermost region and as shaded as the uniform surroundings in its interior region. Examples of the various stages of condensed bodies may be found in the video photographs. Occasionally there were dark bodies with a lipid density so low that they remained dark at large sizes. The absence of brightening in the earlier study of dark bodies in PC/water systems may have been due in part to the fact that those samples were only 20 μm thick [4]. Bubbles of expanded phase arising in condensed phase were discovered only in the present study. We refer to the photographs for the darkening of initially bright bubbles with increasing size which again results from the effects of phase contrast. The contrast reversal of dark bodies, single or overlapping each other, was sometimes used for a rough estimate of their lipid density. We found the lipid density of the condensed phase minus that of the surrounding expanded phase to be of the order of 3% wt./wt.

Bodies of condensed phase usually rested on the bottom of the sample cell. An exception were the dark bodies just precipitated from expanded phase with diameters below 5 μm. They moved about rapidly and in general fused without delay with other bodies.

Bubbles of expanded phase moved upwards and fused with each other or the outside of the body of condensed phase in which they floated. They diffused much more slowly than dark bodies of equal size. This suggests that the viscosity of the condensed phase is markedly higher than that of the expanded phase.

3.4 *Photographs and their interpretation*

Two series of six video photographs each are shown to illustrate the existence of a lower consolute point and to locate the critical temperature. In the first series, figure 2, the sample was cooled from 74°C to 17°C after one hour of annealing at the very high initial temperature (pictures on the left) and immediately reheated to 55°C (pictures on the right). The first picture shows a body of condensed phase at 55°C with a sharp boundary roughly coinciding with the outer edge of the bright ring. The border of the body is diffuse at 25°C and even more so at 17°C. The extremely low visibility of the widened border is a consequence of phase contrast. Reheating produces phase separation with dark bodies and bright bubbles on the sides of the wide border region as shown for 30°C. Later on, at 55°C, one sees a boundary between a condensed phase inside and an expanded phase outside. The double line is an artefact; it does not indicate the presence of one or two vesicle membranes.

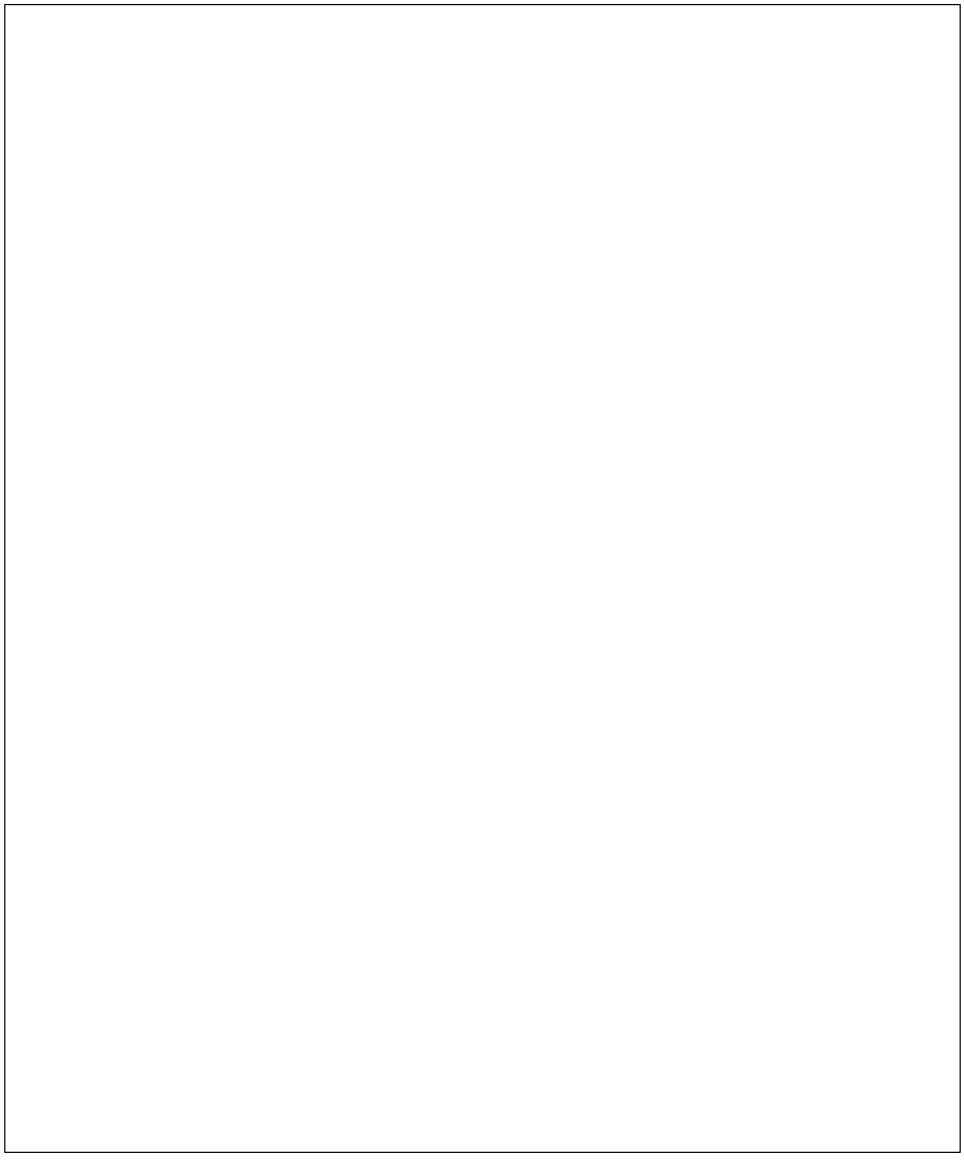

Figure 2. Going through the critical temperature to lower (left) and higher (right) temperatures. See main text for detailed description. The sample is 80 μm thick and consists of spinach DGDG swollen in 20 mN NaCl solution.

We may infer from the sequence of pictures that there is a lower consolute point around 25°C where we see simultaneous segregation of bodies and bubbles on heating. The increasing diffuseness of the border as the temperature drops through the critical point may be expected on the basis of van der Waals or, generally, Ising critical behaviour. The widening of the border seems to be restrained by slow material transport. (It would probably have been completely suppressed without the heat treatment prior to cooling;

see also above.) The lipid density difference between inside and outside of the body reemerges upon heating. In fact, the border sharpens again by the fusion of small bodies of condensed phase with the central body and the opposite effect, the ejection of bubbles of expanded phase.

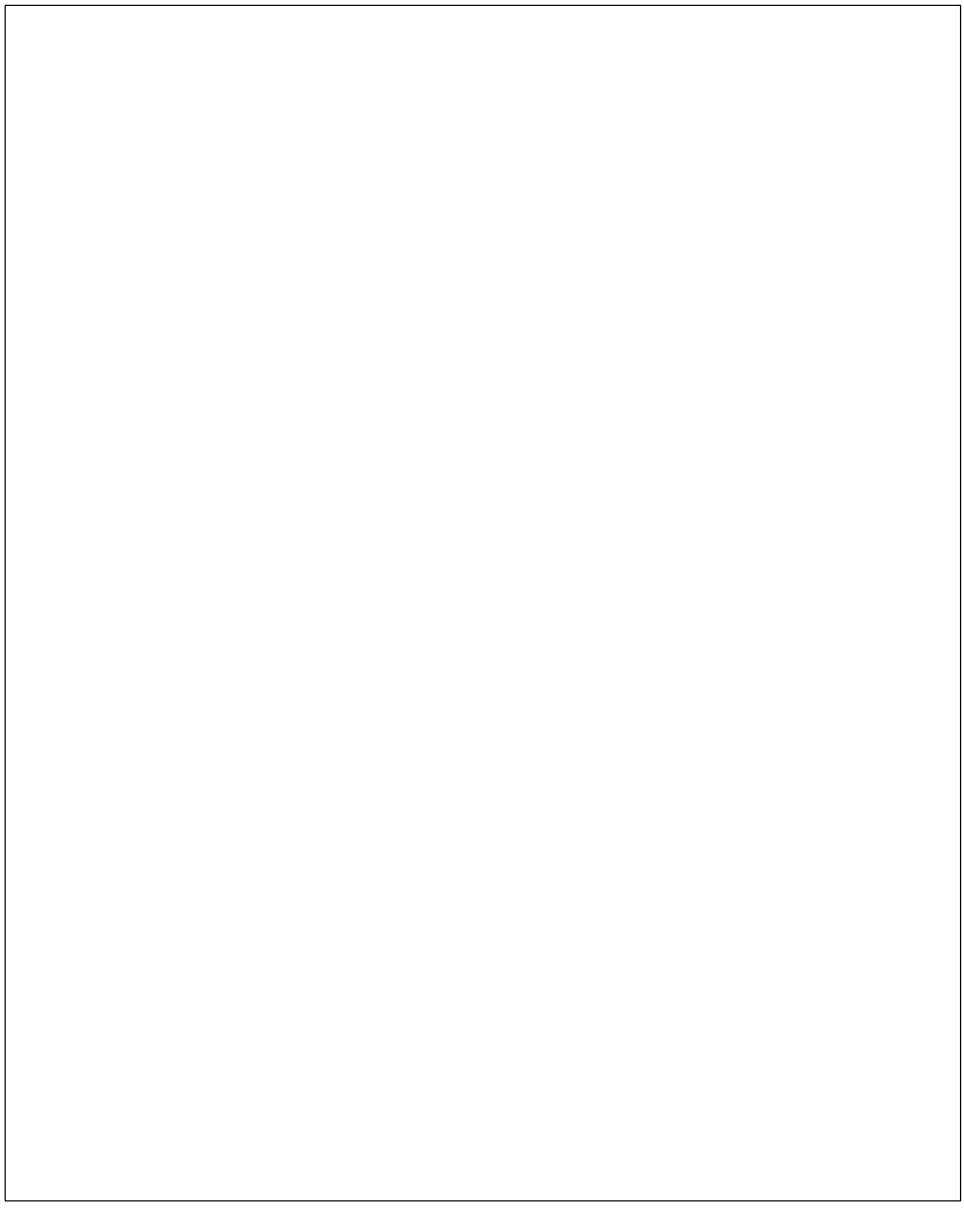

Figure 3. Last stages of 24 h stay near the lower critical point (left) followed by rapid heating (right). See text for details. The sample is 50 μm thick and consists of comfrey DGDG swollen in 10 mM NaCl solution.

The second series of video photographs, figure 3, allows a more precise determination of the critical temperature. The row of pictures on the left was taken at the end of a 24 h period at room temperature. One sees a very weak phase boundary whose end is very slowly retracting to the bottom of the section shown. The last part of the borderline is roughened and on video one notices very conspicuous density fluctuations in the vicinity of the end, especially on its left-hand side.

We think that in these three pictures the lipid/water system is very close to its lower consolute point, both in temperature and lipid density. However, there appear to be weak vertical and horizontal gradients of temperature and lipid density, respectively. The nearness of the critical point and the density gradient are confirmed by the row of pictures on the right. They show the beginning of a phase separation and, on the right-hand side, the precipitation of dark bodies.

These effects may be expected when the temperature rises from the critical point. In the last picture a boundary between condensed and expanded phases is seen in the bottom left corner. One may speculate that it represents a percolation boundary which does not appear at the critical point but only at a higher temperature and replaces a region of interpenetrating phases. The enormous interpenetration is probably a dynamic effect since it is absent in the nearly static case shown on the left of figure 3.

As a curiosity, we show in the last series of video photographs, figure 4, a body of condensed phase sucking in and digesting other bilayer material. The sample had been at 50°C from the start of swelling. The pictures cover only a small fraction of the time during which the body was 'voracious' in this way. The process is easily distinguished from the much more common fusion by two features: First, the objects absorbed are not spherical but either cylinders or 'ropes', both of which are visible in figure 4, and optically denser than the expanded phase. Second, it takes about a minute until an object is digested after ingestion by the body of condensed phase so that the latter looks uniform again. The considerable commotion accompanying digestion may be assessed by comparing subsequent pictures of the voracious body.

The first four pictures of the sequence show the ingestion of a rather wide cylinder. The ropes seen in the last two pictures are breaking loose from the voracious body and finally shrivel (not shown) toward the bottom of the picture. The transition from a residual cylinder to two ropes between the fourth picture and the fifth could not be perceived step by step because of optical overlap with the other cylinder seen in the photograph.

The nature of the absorbed lipid material is unclear. We think that the bilayers are in an intermediate state between practically flat and fragmented. Sometimes the material comes off a myelin cylinder of high lipid density like thick pieces of bark before it feeds a rope. The ropes are slightly lumpy and ca. 5 μm thick. They can last several minutes, keeping up transport all the time with a velocity of the order of one rope diameter per second.

4. Discussion

Let us offer a model for the fragmented (or nodular) bilayer state with a lower consolute point. It is inspired by the micrographs of knotted sticks obtained in cryo-transmission electron microscopy of the dispersive phase of PC bilayers in water [3]. We identified the dispersive phase with the expanded phase and the dark bodies with the condensed phase.

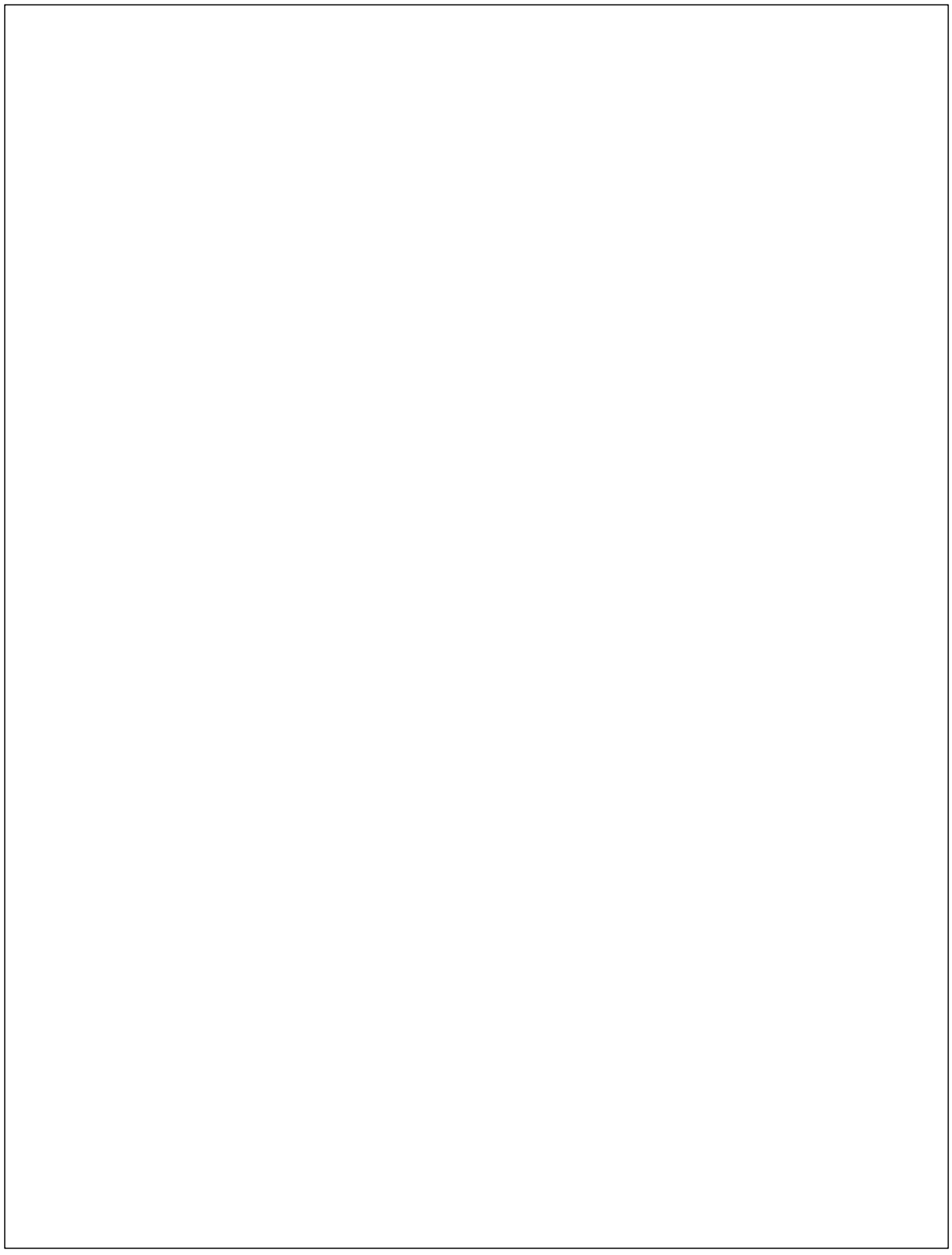

Figure 4. A voracious body of condensed phase in action. See text for details. The sample consists of spinach DGDG swollen in 10 mM NaCl solution.

While the knotted sticks are scattered in the expanded phase, they are assumed to form an irregular network in the condensed one. The knotted sticks may be connected by simple branchings, parting either at a knot or at a tubelike constriction. The bending energy of a

knotted stick is likely to be negative as compared to the flat bilayer state. However, the bending energy of the hemispherical stick ends will be positive. This would explain the tendency of the knotted sticks to attach to each other by means of branchings. Moreover, in the expanded phase, one may expect the sticks to become longer with decreasing temperature.

Based on this tentative model we can try to rationalize two of our observations. First, the absence of phase separation at low temperatures might be due to a large length of the sticks. In the resulting system of intertwining stiff chains, all that is left may be a thermodynamically irrelevant percolative transition. Second, the inertness of 'mature' bodies of condensed phase with respect to dissolution (and possibly fusion) at $T < T_c$ might result from the binding of practically all loose stick ends to the network in the course of time.

Our experiments prove that there is a lower consolute point in DGDG/water systems near room temperature and at a lipid density of very approximately 1%. They were not systematic enough to detect any dependence of T_c on time as a consequence of chemical decomposition. Likewise, we did not try to find differences in critical behavior between the two DGDG variants, one extracted from spinach and the other from comfrey.

It should be mentioned that dark bodies and bright bubbles sometimes appeared on heating, already several degrees below T_c. These observations were preceded by incubation periods of 15 min or longer at even lower temperatures down to 5°C. The preliminary data suggest that the early appearance results from a delay of equilibration of the fragmented state. An important parameter in this respect could be the number of lipid molecules per knot. Any deviation of this number from a temperature dependent equilibrium value might render the knotted sticks more reactive so that branchings and thus phase separation are promoted.

5. Conclusion

One may wonder if the fragmented bilayer state is metastable, intervening on the way from the flat bilayer to a stable bilayer cubic phase, like the inverted hexagonal phase of some phosphatidylethanolamines (PE) [8]. It seems possible that a cubic phase is the state of minimum bending energy because in a sense it consists of passages only. The idea that the fragmented state is not the stable one is supported by the finding that PC bilayers can rupture to transform into a phase of high lipid density ($\approx 30\%$) which could well be cubic [9].

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