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Phase transitions of bipolar lipids of thermophilic archaebacteria

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The plasma membrane of *Sulfolobus solfataricus*, an extreme thermophilic archaebacterium, is characterized by unusual bipolar lipids arranged in a single monolayer. They are based on two C_{40} ω-ω' biphytanyl residues, with up to four cyclopentane rings per chain, linked to either two glycerols (symmetric lipid) or to one glycerol and one branched-chain nonitol (asymmetric lipid). In this work we present a comparative calorimetric study of the symmetric and asymmetric lipid at various degrees of hydration. The results are compared with X-ray diffraction data. The thermotropic properties of the asymmetric lipid are very complex ones, showing two transitions in the dry state and at least two in the hydrated one. The low enthalpies displayed by the higher-temperature transitions indicate lipid polymorphism associated with temperature-dependent hydrogen bonds between the nonitol polar heads, while the lower-temperature transitions are mainly associated with structural changes of the hydrophobic core. The symmetric lipid exhibits a much simpler behaviour, being arranged in a lamellar structure with a single melting point. It shows, however, phase separation between hydrated and non-hydrated lipid domains, which allows some general considerations to be made on the non-random way water is absorbed and released in the interlamellar spaces.

Introduction

The elucidation of mechanisms underlying the thermostability of thermophilic bacteria enables one to identify the physico-chemical strategies that a cell employs in adapting to thermal stress. These strategies are also relevant to biological evolution, since thermophilic archaebacteria might have been among the earliest inhabitants of the earth. *Sulfolobus solfataricus* (previously named *Caldariella acidophila*) is an atypically walled thermophilic archaebacterium [1,2]. It contains no peptidoglycan, but is enveloped by a cytoplasmic membrane which is further overlaid by a polyhexagonal array of protein subunits 13 to 15 nm in diameter [3]. A pH gradient exists across the membrane, which approximates 3.5 to 4.5 pH units, the internal pH value being around 6 and the external one between 1.5 and 2.5 [1]. All membrane lipids of archaebacteria so far identified are characterized by unusual structural features, which can be considered to be specific taxonomic markers of this group of microorganisms [4]. In fact while all living organisms so far known have membranes based on ester linkages, formed by the condensation of alcohols and fatty acids, archaebac-

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teria have lipids based on ether linkages. These molecules are formed by condensation of glycerols or more complex polyols with isoprenoid alcohols containing 20, 25 or 40 carbon atoms. In *Sulfolobus solfataricus* the lipids are bipolar, as shown in Fig. 1, and based essentially on the tetraethers. These tetraethers can be divided into two classes; the first R = H, may be simply called glycerol-dialkyl-glycerol tetraether (GDGT). The structural organization of the second class of molecules in which R = $C_6H_{13}O_6$ is similar, even though a more complex branched nonitol replaces one of the glycerols; these molecules have been named glycerol-dialkyl-nonitol tetraethers (GDNT) [5]. These tetraether lipids constitute the skeleton of the complex membrane lipids, i.e. phospholipids, glycolipids, sulfolipids. The C_{40} components of these lipids differ in the additional feature of containing up to four cyclopentane rings per chain; the degree of cyclization in the biphytanyl component is sensitive to the environmental parameters such as temperature. In fact is has been shown that the extent of cyclization increases when *Sulfolobus solfataricus* is grown at increasing temperatures [6].

The organization of these membrane lipids must be very peculiar, in view of the enormous pH gradient that the cell must withstand and of the very high temperature of growth (between 70 and 90°C). We have shown that the plasma membrane of this microorganism comprises a simple monomolecular layer based on bipolar lipids [4,7]. In this paper we present a study of the thermotropic properties of GDGT and GDNT molecules as a first approach to the complex membrane lipids.

The symmetric lipid behaves quite simply, being arranged in a lamellar structure at any degree of hydration. It displays a melting transition [8,9] whose temperature depends on the number of cyclopentane rings. Moreover, differential scanning calorimetry (DSC) measurements on biphytanyl tetraether glycolipids from another archaebacterium, *Thermoplasma acidophilum,* which has a lower degree of cyclization, have also shown a lower transition temperature (around −20°C) [10,11]. Experiments in open sample pans give some general information on the mechanism by which water is released by the sample and on the nature of the melting transition. The thermodynamic meaning of this procedure is discussed in the next section.

The thermotropic behavior of the asymmetric lipid is a very complex one, showing several transitions. A very low enthalpy is associated with most of them. The behavior is similar to that observed, for instance, in phosphatidylethanolamine on proceeding from the lamellar to the hexagonal phase [12]. Water is shown to play a fundamental role in determining the kind and the temperature of the transition. Indeed recent X-ray diffraction experiments [13] showed the presence of complex polymorphic phases dependent on temperature and degree of hydration. The calorimetric behavior associated with these transitions is presented in this paper.

Materials and Methods

Sulfolobus solfataricus strain MT-4 was isolated from an acid spring in Agnano, Napoli. The bacterium was grown as previously described [3] at 87°C. The details of lipid extraction can be found elsewhere [5]. The composition of the lipid native mixture was determined. The results are listed below. The values in the parentheses represent the number of pentane rings per each chain (see Fig. 1).

Symmetric lipid: (2 + 2) 20.5%; (3 + 3) 28.6%; (2 + 1) 6%; (3 + 2) 41.3%; (3 + 4) 3.6%.

Asymmetric lipid: (2 + 2) 20.1%; (3 + 3) 31.7%; (3 + 2) 45.2%; (3 + 4) 3%.

Calorimetric measurements were performed on the dry and on the hydrated sample, using a differential scanning calorimeter (Mettler TA 3000). Determination of the transition points and of the enthalpy production was generally performed on line, by means of a microprocessor connected to the calorimeter. The scanning rate was usually 2 K/min and the sensitivity as indicated in the thermograms. The lipids were dissolved in chloroform and directly evaporated into the aluminium pans, which were previously weighed on a Mettler M3 microbalance. To remove any residual trace of bound water the aluminium pan was left in an oven at 85°C for at least 1 h, and the dry weight was then determined. Since dessication was not performed under vacuum conditions, some residual trace of bound water could still remain.

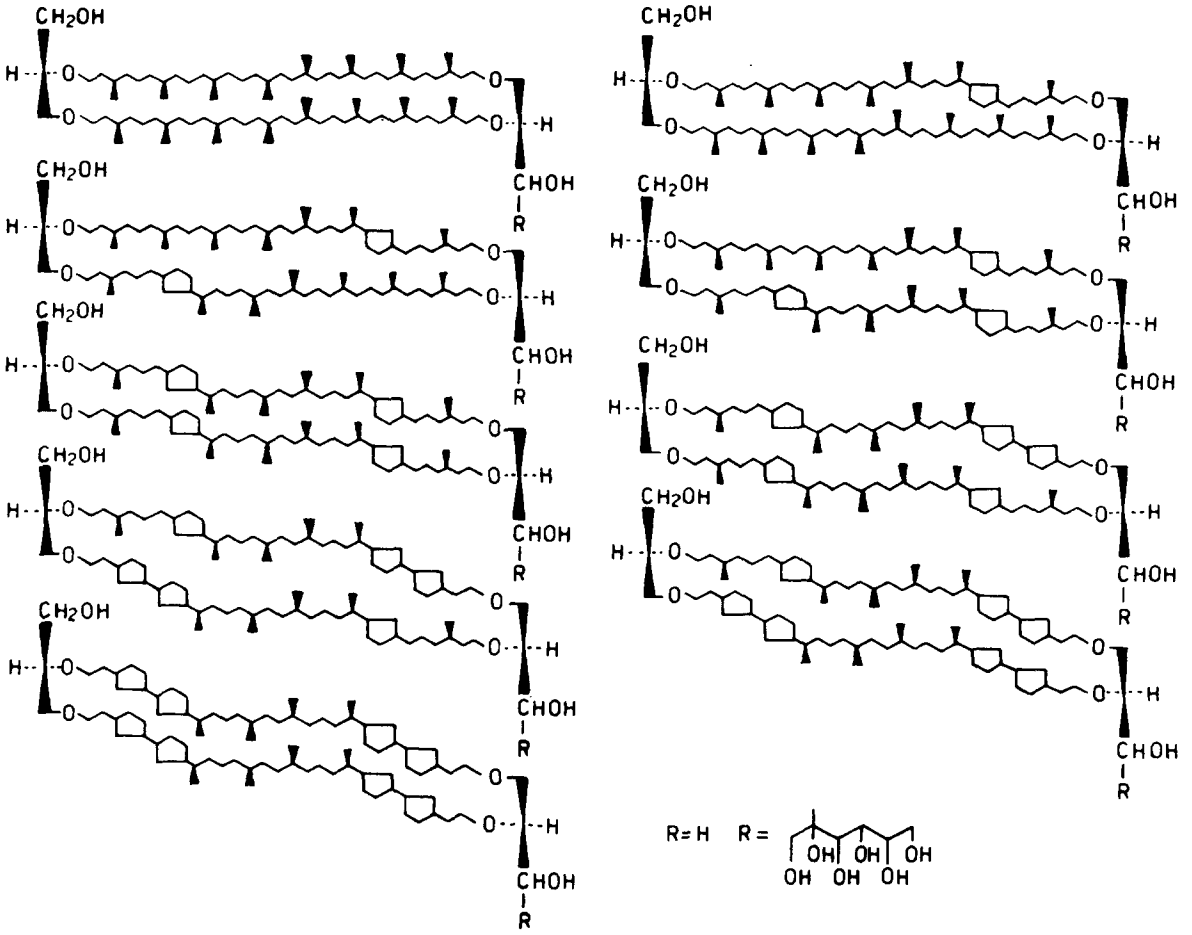

Fig. 1. Structures of isoprenoid ethers, backbone of complex lipids of *Sulfolobus solfataricus*.

Measurements on the hydrated lipid were performed according to the following procedure. The aluminium pan containing the dry lipid was placed under a glass bell containing a small amount of water layered on the bottom. Vacuum (30 mm Hg) was then applied in order to have a saturated aqueous vapor chamber. The system was then left at 25°C for at least three days in order to induce a very slow hydration of the lipid. Such a procedure ensured very reproducible results at intermediate hydration values. No substantial change in water absorption was detected within an interval of temperature of few degrees in the hydration procedure. The amount of water absorbed was then measured by weight. The sample was successively scanned several times and the progressive loss of water followed by weighing the sample after each run. When the desired amount of water was obtained the aluminium pan was sealed, at intermediate hydration values. The values collected in Table I are obtained with such a procedure. In any case all the facts reported are based on repeated observations.

The lamellar phase of GDGT, at partial hydration, showed the simultaneous presence of dry and hydrated domains. Therefore measurements of Fig. 2a–g were carried out in open pans, to study how one peak is merging into the other. This procedure is justified by the finding that the transition peaks occur at $T < 25°C$, where the lipid sample behaves as a closed thermodynamic system. In fact negligible water loss, observed as a drift in the baseline, occurs below 25°C. This is clearly indicated by Fig. 2p, where a saturation in the drift of

the base line is displayed at such temperature values. Thus, besides avoiding manipulations of the lipid among the runs, the open-pan procedure allows also getting important information on the mechanism by which water is lost by the lipid, as will be explained in the Discussion section. In a few experiments water was directly added to the dry lipid sample. The enthalpy values of these samples were, within experimental error, equal to those obtained with the slow hydration procedure at full hydration. Single measurements of enthalpy can be affected by deviations of up to 25%, due to the broadness of the peaks. In fact because of this behaviour there is a certain arbitrariness in the choice of the baseline in the peak integration procedure. For this reason we have performed evaluation of the enthalpy with both base lines indicated in Figs. 2b and 2c.

Analysis on the thermogravimetric balance (Mettler TG 50 thermobalance system) allowed determination of the rate of water loss from the fully hydrated sample.

Results

Symmetric lipid GDGT

The symmetric lipid, hydrated as described in Materials and Methods, absorbed a fixed quantity of water, corresponding to 2.8% of its dry weight. These water molecules represent the firmly bound water, in that the thermogram does not give any peak of melting of free water. Such amount of water corresponds to two molecules of water per lipid molecule; i.e. one water molecule per OH group.

Figs. 2 a–2g show the overall sequence of thermograms performed by rescanning a sample of 7.9 mg from 40 to −10°C in an open pan at a rate of 2 K/min. It clearly indicates the presence of two peaks, corresponding to the almost fully hydrated state (Fig. 2a) and to the almost completely dry one (Fig. 2g). The other thermograms performed at intermediate values of water content, represent the phase transitions corresponding to simultaneously coexisting dry and hydrated domains. Notice the asymmetry of the two peaks, the hydrated one being much sharper than the dry one. In the fully hydrated state the peak is around 25°C, while in the dry state, the maximum value

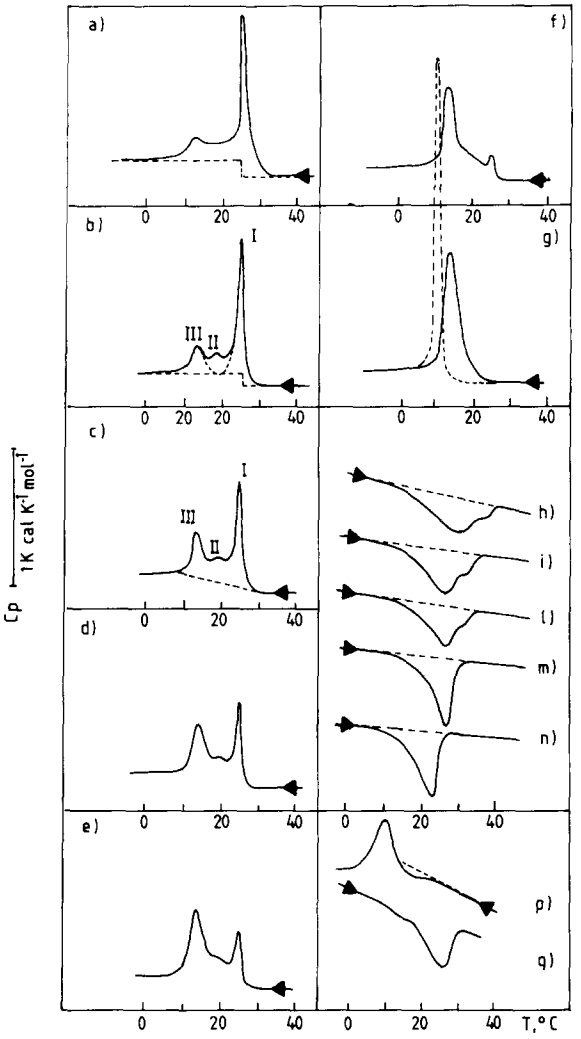

Fig. 2. A series of thermograms in a pattern of decreasing (a–g) and increasing (h–n) temperatures. Successive scans of 7.9 mg symmetric lipid in an open pan are shown. The arrows indicate the direction of the scans. Scanning rate 2 K/min. The base lines employed to determine the area under the peaks are indicated in (b) and in (c). The dotted line of (g) and peak (n) correspond to the sample dried in an oven at 80°C for 5 min. Peaks in (p) and (q) correspond to a 10.9 mg sample previously subjected to several scans and manipulations. This sample, which is presumably less ordered, does not show, at intermediate hydration values, separation between dry and hydrated peaks. Notice that also the transition temperatures are lowered. Further details are given in the text.

of the peak is at 11°C. After seven scans the system is almost completely dry.

The resolution of peaks superposition was performed with the procedure illustrated in Fig.

2b. Peak III has been reconstructed considering it symmetrical and supposing that the baseline does not vary significantly upon the transition. By contrast peak I was integrated by choosing the asymmetrical baseline shown in Fig. 2b. The difference between the total area and that of the two peaks gives the enthalpy of peak II. The latter has been determined also directly and the two values are equal within the errors. Notice that the difference in baseline is likely due to a glassy transition present at lower temperature (cf. Fig. 2q). Such kind of transition is also present in the asymmetric sample (cf. Fig. 4b) and in other tetraether lipids [9–11]. Therefore, the base-line indicated in Fig. 2c has also been used. Plotting the enthalpy of the peak I as a function of the peak III one gets in a wide range of hydration the linear relationship shown in Fig. 3, while the enthalpy of the intermediate peak remains almost constant as shown in the same figure. Analysis of the data indicates that the behaviour reported in Fig. 3 is independent, within a constant, of the choice of the two base-lines.

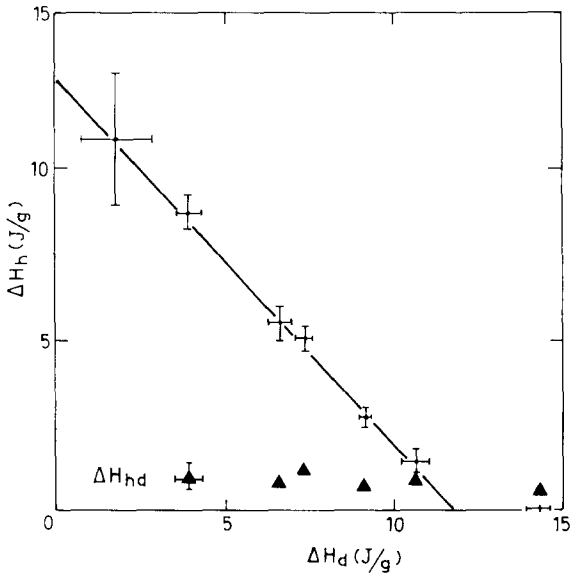

Fig. 3. The enthalpy of the hydrated peak ΔH_h as deduced from the thermograms of Figs. 2a–2g, is plotted against the enthalpy of the dry one ΔH_d. Both base lines shown in Figs. 2b and 2c give rise to a linear relationship. The bars indicate the errors in the enthalpy evaluation once the base line (in this case (c)) has been chosen. The triangles indicate the enthalpy of the intermediate peak ΔH_{hd}; the typical error is indicated on the first point.

The two peaks obtained by cooling the lipid are clearly separated. By contrast if the same set of measurements is repeated by warming the system, hydrated with an identical procedure, much less separation between dry and hydrated peaks is detected, and both peaks are shifted by a different amount towards higher temperature as Figs. 2h–2m indicate. This behaviour is only slightly affected by scan rate in the range 2–5 K/min. Since the peaks are now in the evaporation region, the system cannot be considered closed from the thermodynamical point of view and therefore no quantitative analysis can be performed.

Water evaporation explains in part the difference in the shape of the heating and cooling scans. However, the main reason is related to the metastability of the peaks and their consequent splitting during the cooling run. In fact by leaving the dry sample in an oven for 5 min at 80°C the peak shown by the dotted line of Fig. 2g is observed in the cooling procedure while in the heating one the peak shown in Fig. 2n appears. The transition temperature differs by about 13 K. By contrast when the lipid has undergone several temperature runs and manipulations (i.e. at lower order and purity of the sample), the two peaks are not separated and no relevant difference in shape upon cooling and warming is detected as Figs. 2p and 2q show. The same considerations apply to closed pans.

Asymmetric lipid GDNT

When the sample was hydrated using the slow procedure described in Materials and Methods, it absorbed an amount of water up to 26% of its dry weight. This figure corresponds to 21 molecules of water per molecule of lipid. To summarize the complex behaviour of this lipid, data are collected in Fig. 4 and Table I. Fig. 4a is the phase diagram deduced by X-ray diffraction analysis [14], performed above 15°C. The phases observed are a rectangular phase P, a cubic phase, Q, and an hexagonal phase H. In all of them the hydrocarbon chains take up a disordered conformation. The region indicated with N corresponds to an unidentified phase (or phases) with rigid chains. The vertical lines indicate the values of hydration at which the calorimetric runs, reported in Fig. 4b, have been performed. The points indicate the oc-

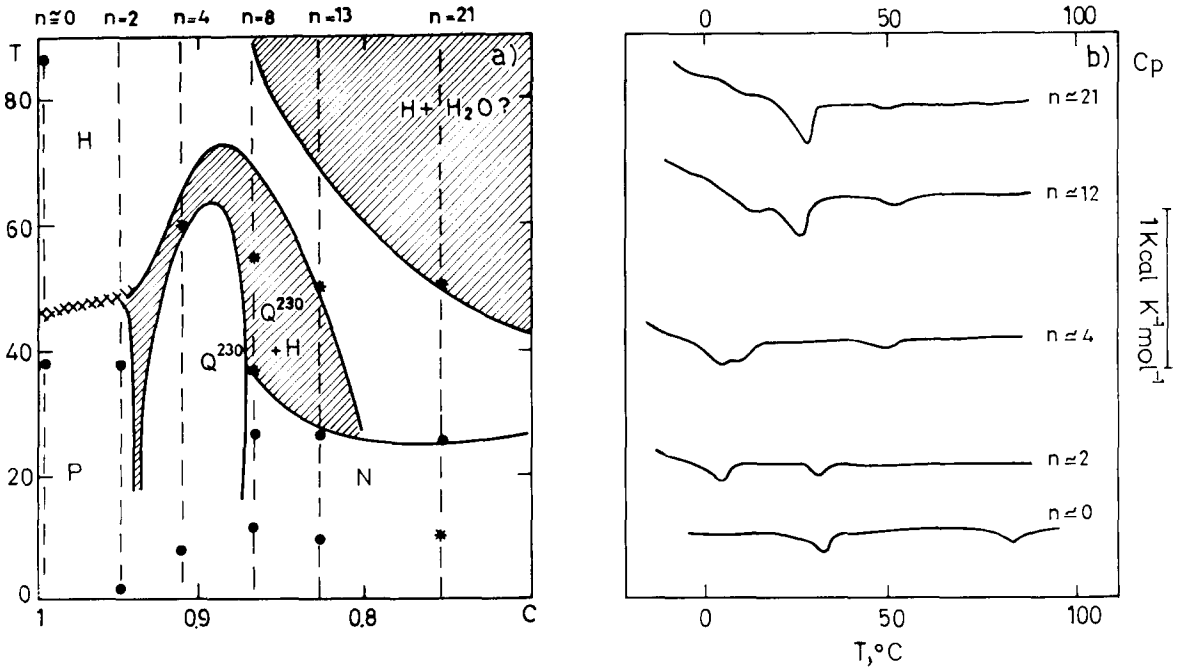

Fig. 4. (a) Phase diagram of the system GDNT-water. The concentration c is the ratio lipid/lipid + water, expressed by weight. The vertical lines correspond to the calorimetric scanning performed at the indicated values of n (number of water molecules per lipid). Circles indicate the temperature at which calorimetric peaks are observed. The corresponding enthalpies are given in Table I. The asterisks indicate irregularities in the thermograms (see text). (b) Thermograms of 10.8 mg asymmetric lipid at the indicated values of n. Scanning rate 2 K/min.

currence of heat absorption peaks whose enthalpy is given in Table I. The asterisk indicate irregularities in the thermograms with enthalpy production (if any) below the instrumental sensitivity (0.5 J/g). The temperatures at which such kind of discontinuities are observed is quite reproducible.

TABLE I

CHARACTERISTICS OF THE PHASE TRANSITIONS OF ASYMMETRIC LIPID AS A FUNCTION OF THE HYDRATION NUMBER (i.e., THE NUMBER OF WATER MOLECULES PER LIPID, n)

The enthalpy production ΔH and the transition temperature T are given as functions of the lipid hydration number for the observed transitions. The asterisk indicates the presence of a transition characterized by a very small enthalpy production, which was not sufficiently larger than the experimental error to allow quantitative determination. The accuracy in the enthalpy determination is rather low (+25%) owing to the broadness of the peaks and to a certain arbitrariness in the choice of the baseline.

n	ΔH_1 (J/g)	T_1 (°C)	ΔH_2 (J/g)	T_2 (°C)	ΔH_3 (J/g)	T_3 (°C)	ΔH_4 (J/g)	T_4 (°C)
21	*	11	5	25		* 50°C		
15	1	11	6	26		* 50°C		
13	2	11	7	26		* 50°C		
8	3	9	4	26		* 35–55°C		
4	3	9	–	–		* 57°C		
2	3	6	–	–	1	35		
1.5	1.5	4	–	–	2	35	0.5	85
1.1	1	2	–	–	2	35	2	86
0	–	–	–	–	2	35	2	88

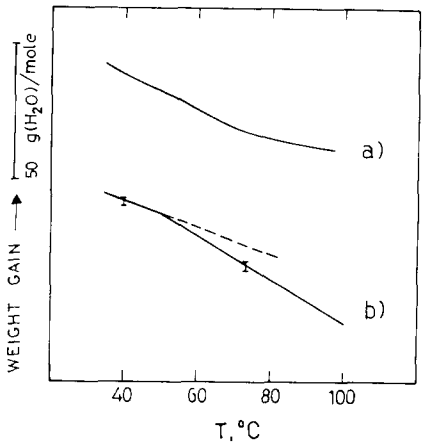

Fig. 5. Water loss per mole of (a) symmetric and (b) asymmetric lipid versus temperature, as deduced by thermogravimetric measurements. The break around 52°C in the curve (b) indicates a change in the organization of the polar heads and corresponds to the appearance of a H_2O phase (cf. Fig. 4a). Notice the opposite trend of the two curves at high temperature. Scanning rate 2 K/min. The sensitivity of the measurement is indicated by the bar.

Thermogravimetric measurements

Thermogravimetric measurements were performed, at a scanning rate of 2 K/min, in the range 35–95°C, both on the symmetric and on the asymmetric lipid fully hydrated. The results indicate in the case of the symmetric lipid a constant water loss of 0.6 g (H_2O)/mol per K up to 70°C (Fig. 5a). By contrast a significant break in the straight line occurs around 52°C in the asymmetric lipid (Fig. 5b). The slope of the two lines are 0.5 g(H_2O)/mol per K and 0.8 g(H_2O)/mol per K, respectively. It is expected that both thermogravimetric curves tend to a saturation while approaching lower temperature values.

Discussion

Symmetric lipid

Data shown in Fig. 2a–g indicate the presence of two kinds of domains, either with two water molecules per lipid, or without any water at all. Peak III around 10°C represents the dry domain (dd), while peak I around 24°C represents the fully hydrated one (hh). The small peak around 19°C might thus represent the intermediate state with a single water molecule per lipid (hd). The linear relationship between the enthalpies of the peak I and those of the peak III shown in Fig. 3 suggests a model for the mechanism of dehydration of the system, for those hydration values where such a linear relationship holds. Indeed Fig. 3 seems to imply a direct transition of fully hydrated lipid molecules (hh) into dehydrated ones (dd):

$$hh \rightarrow dd$$

which implies a process where two molecules of water are eliminated at once. By contrast the two-step process

$$hh \rightarrow hd \rightarrow dd$$

seems to be excluded by the fact that the intermediate peak remains constant.

There is no reason why the two water molecules should be lost by the two polar heads of the same lipid chain. We suggest instead that this process is a consequence of an ordered lamellar structure of the lipid in which homologous (h-h, d-d) polar heads are facing together as indicated in Fig. 6. The dehydration phenomenon probably occurs in the boundary regions between hydrated and dehy-

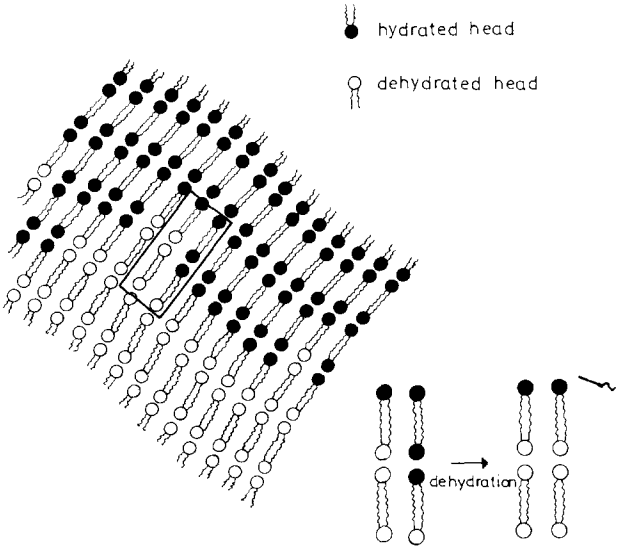

Fig. 6. Schematic drawing of the rippled state of GDGT after the melting transition. The inset indicates the evaporation process by which two water molecules are lost at the boundary region between dry and hydrated domains, where half-hydrated molecules are present.

drated domains as shown in the inset of Fig. 6. Indeed such boundary regions correspond presumably to places where the hydrogen bonds are subjected to higher stresses. Since this ordered structure is not destroyed by evaporation, then the elementary step of the dehydration involves two different lipid molecules according to the reaction (see inset of Fig. 6)

$$hh + hd \rightarrow hd + dd$$

Therefore the fraction hd of the lipid, which remains constant for a wide range of hydration values (see Fig. 3), plays the role of a catalyst of the dehydration reaction. Of course this mechanism fails at the extremes of full hydration and full dehydration. Support to this model is provided by X-ray diffraction data which show a planar lamellar arrangement of lipids both in the dry and in the hydrated state. However, this order is apparently destroyed after the melting transition [13]. We may resolve this point by assuming that in the fluid state molecules are still stacked in lamellae, but in rippled surfaces, as Fig. 6 shows, which give rise to a weaker diffraction pattern. Indeed ESR studies [14] indicate that after the melting transition the order parameter is still high, as expected for such a kind of arrangement.

As an immediate consequence of this model, in a wide temperature range water should be lost proportionally to the constant fraction hd of the lipid and not to the actual water content. This is indeed the result of the thermogravimetric measurements shown in Fig. 5a, up to a temperature around 75°C; at $T > 75$°C a different mechanism of water loss seems to occur, since the rough lamellar structure is destroyed at such temperatures [14].

It is worthwhile to underline that, at variance with usual lipids, the transition temperature of the dry compound is lower than that of the hydrated one. Since the enthalpy production is somewhat smaller in the hydrated case ($\Delta H_h \simeq 13$ J/g; $\Delta H_d \simeq 15$ J/g) the entropy variation of the dry system must be higher. This can be explained on the basis of the following considerations. Hydrogen bonding between neighbouring glycerols imposes a highly ordered lamellar structure. Hydration relaxes the steric constraints allowing a higher mobility of the molecules or, more precisely, a lower order parameter, as indeed observed in EPR measurements [14]. The melting transition of the hydrated system is thus expected to imply a lower entropy variation, as required by thermodynamics.

Asymmetric lipid

The phase diagram of the asymmetric lipid shown in Fig. 4(a) is quite complicated; we nevertheless discuss some general considerations. For a lipid with flexible chains, whenever a transition occurs between two regions, and one of them contains two phases, calorimetric peaks are barely observed or not observe dat all because the heat is spread over a wide temperature range. By contrast the transition occurring at lower hydration between the two one-component regions P and H clearly shows a calorimetric peak, even though characterized by a very small enthalpy production ($\simeq 2$ J/g).

When changes in the hydrophobic core from a rigid (N) to a flexible structure (H) are involved, higher values of heat absorption peaks are observed, indicated by the circles in Fig. 4(a). However, such enthalpy production is about one third of that of GDGT and at least one order of magnitude lower than that of usual monopolar lipids undergoing a gel-liquid crystal transition [15]. This fact might suggest the occurrence of a transition of part of the chain, also because an additional lower temperature peak has been observed in the rigid phase. Alternatively these two peaks might indicate the presence of domains with a different degree of hydration, as already found for the GDGT sample. Notice that formation of domains in GDNT planar lipid membranes has also been detected [16].

The transition from the homogeneous hexagonal phase H to phase H plus water, observed at high hydration, is clearly indicated by the change in the trend of the thermogravimetric curve around 52°C (Fig. 5b). In fact part of the water, no longer linked to the lipid structure, can more easily leave the system.

At low hydration the transition from the rectangular (P) to the hexagonal phase (H) involves mainly changes in hydrogen bonds between the polar heads, since chains are flexible in both phases. This fact accounts for the small enthalpy

observed (cf. Table I). However, ESR measurements [14] have shown that the rotational motion of the chains is affected by this transition even in the deep hydrophobic core. In contrast to the hydrated phase, the dry state does not seem to imply the occurrence of stiff chains even at low temperature (down to $T = 0°C$), possibly because of the higher steric constraints imposed by the nonitol head groups. Saturation transfer ESR studies [14,17] have shown a strong immobilization of the outermost hydrophobic portion of GDNT not only with respect to GDGT but also with respect to an usual egg phosphatidylcholine lipid. In fact, in this very low hydration region the correlation time is up to three orders of magnitude higher than that of more common lipids.

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