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EFFECT OF ISOPRENOID CYCLIZATION ON THE TRANSITION TEMPERATURE OF LIPIDS IN THERMOPHILIC ARCHAEBACTERIA

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The plasma membrane of *Caldariella acidophila*, an extreme thermophilic archaebacterium, is characterized by unusual bipolar lipids. 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 to one branched-chain nonitol (asymmetric lipid). When *C. acidophila* is grown at various temperatures, these lipids show a degree of cyclization of the biphytanyl components which increases as the environmental temperature increases. The rôle of cyclization in determining the temperature adaptation is studied on three lipid samples presenting four, five and six cyclopentane rings per molecule, respectively. Differential scanning calorimetry on the dry asymmetric sample as well as conductance and capacitance measurements on black films have been performed. Both sets of measurements indicate the presence of thermal transitions, three in the hydrated compounds, two in the dry system. The latter are shifted towards higher temperature values as the number of cycles increases. Calorimetric measurements show that two of these transitions are strictly related to the presence of nonitol-containing polar heads. In fact, only a single 10-fold higher transition is detected in the homologous lipid bearing two glycerol polar heads, in the dry as well as in the hydrated form. It is suggested that the two higher-temperature thermal transitions, observed on warming the sample, are induced by the breaking of hydrogen bonds between the nonitol-containing polar heads. By contrast, the lower temperature transition, present only in the hydrated compound and similar to that exhibited by the symmetrical sample, is due to a partial melting of the hydrophobic core. The large change in capacitance observed near the higher transition points by lowering temperature would thus correspond to variations in the dielectric constant due to formation of hydrogen bonds.

Introduction

In previous papers [1,5], we have described the lipid structures of *Caldariella acidophila* [6] (recently named also *Sulfolobus solfataricus* [7]), an extreme thermoacidophilic archaebacterium [8,10]. The complex membrane lipids of this microorganism are amphipathic molecules which, irrespective of complex lipid structures, have two polar heads (two glycerol or one glycerol and one nonitol), linked together by two C_{40} biphytanyl chains, that differ from each other by the feature of up to four cyclopentane rings (Fig. 1).

C. acidophila can be grown at temperatures between 75 and 89°C with reasonable facility. As the growth temperature is increased, all the lipids show increasing portions of the tri- and tetracyclic biphytanyls (i.e., with six to eight cyclopentane rings per lipid molecule) largely at the expense of the acyclic and monocyclic ones (Ref. 11 and De

Rosa et al., unpublished data). An observation of particular significance is that the glycerol-dialkyl-nonitol tetraether-containing lipids show more extensive cyclization with respect to the temperature increase than the glycerol-dialkyl-glycerol tetraether-containing lipids.

Membranes based on these tetraethers (which, for simplicity, will be called symmetric (Fig. 1a) and asymmetric (Fig. 1b) lipids, referring to their polar headgroup), are organized as covalently bound bilayers, in which each molecule, when fully stretched, anchors the two polar heads to the inner and outer faces of the membrane array [12]. However, because of its biosynthesis [4], such a membrane cannot be modified, in adaptation to higher temperature environments, by the same means as those open to eubacteria with bilayer membranes based on glycerol diesters. Increases in saturation degree and chain length [13,14] and/or the incorporation of specially bulky end-groups in the fatty acids [15], are indeed observed in the latter organisms. The primary effect of such changes is to raise the transition temperatures (melting points) of the layered assembly, and a similar effect can be achieved in the tetraether structure by introducing the observed cyclization into the biphytanyl chains. Each cyclopentane ring

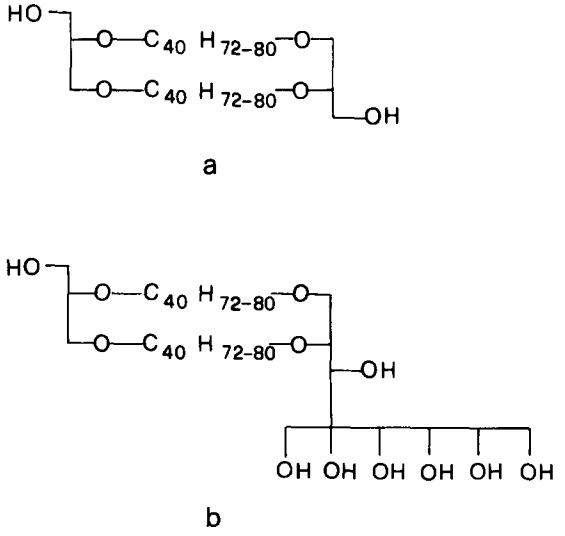


Fig. 1. Structures of (a) glycerol-dialkyl-glycerol tetraethers (symmetric lipid) and (b) glycerol-dialkyl-nonitol tetraethers (asymmetric lipid). Complex membrane lipids of *C. acidophila* are based on these structural types.

in the chain could decrease the available modes of flexing and of rotating and increase the inertial moments of the molecules. The detailed mechanism of such a phenomenon can be better elucidated in a model system.

Recently, we have succeeded in obtaining black films from the crude glycerol-dialkyl-nonitol tetraether mixture [16,17], while we could not form black membranes with the symmetric compound. We have shown that such a membrane is composed of a single layer lacking a mid-plane region [18]. In this paper, we shall present data concerning temperature-induced transitions in the three most abundant molecular species of the asymmetric lipid (72% (w/w) of the glycerol-dialkyl-nonitol tetraether fraction). They are characterized by different degrees of cyclization in the alkyl component, corresponding to four, five and six cycles per lipid molecule (see Fig. 2), which are present in the native lipid mixture from *C. acidophila* grown at 87°C, in a ratio of 20, 32 and 20% (w/w), respectively. Henceforth we shall denote these compounds as 4-, 5- and 6-asymmetric lipids.

Differential scanning calorimetry on dry asymmetrical samples and high-precision specific capacitance and conductance measurements on black membranes are presented. These measurements, although performed in very different experimental situations, are quite well correlated, indicating the presence of two transition points, whose transition temperature increases on increasing the number of cycles. Such transition temperatures, in turn, are shifted towards higher values in 4- and 5-asymmetric lipid black membranes.

Measurements on the hydrated native mixture reveal the presence of a further transition at a lower temperature. Comparison with the thermotropic behaviour of the symmetrical lipid, where only the latter transition is present, indicate that in such a transition the hydrophobic core of the membrane is involved. By contrast, the two higher-temperature transitions, showing a much lower enthalpy production, are strictly related to the presence of the nonitol-containing polar headgroup.

From these results, it can be seen that *C. acidophila* lipids constitute a very informative model system. In addition to its peculiar feature of being the unique model of a monolayer black

membrane, this system may provide information on membrane structure and dynamics over a wide range of temperatures not readily obtainable in the native plasma membrane.

Materials and Methods

Micro-organism and culture conditions. *C. acidophila* strain MT-4, was isolated from an acid hot spring in Agnano, Napoli. The bacterium was grown as described in Ref. 6 at 87°C.

Extraction and hydrolysis of lipids. The micro-organism was extracted continuously (Soxhlet) for 12 h with chloroform/methanol (1 : 1, v/v). The total lipid extract (approx. 8% of dry cells) was treated with methanolic HCl for 6 h under reflux and the hydrolysis mixture was dried in vacuo.

Purification of glycerol-dialkyl-glycerol tetraether and glycerol-dialkyl-nonitol tetraether fractions. The hydrolysis mixture was chromatographed on a Silica (Si)-gel column (40 cm, i.d. 10 mm). Chloroform/ethanol (9 : 1, v/v) eluted symmetric lipid fraction (approx. 17.1% of complex lipids) and chloroform/methanol (95 : 5, v/v) eluted the asymmetric lipid fraction (approx. 55.3%).

HPLC resolution of glycerol-dialkyl-glycerol and glycerol-dialkyl-nonitol tetraethers. The symmetric and asymmetric tetraethers, the latter as fully acetylated derivatives, were further resolved into single components by HPLC. The acetylation was performed with acetic anhydride/pyridine (9 : 1, v/v) under reflux for 6 h; the reaction mixture was dried under vacuum and the acetylated compound was purified by Si-gel column chromatography. After HPLC resolution, the acetylated lipid was hydrolyzed with methanolic HCl under reflux for 6 h. HPLC was performed in *n*-hexane/ethyl acetate (6 : 4, v/v and 8 : 2, v/v for the symmetric and asymmetric compound, respectively) using a microporasil column (3.9 × 30 cm, flow rate of 0.5 $ml \cdot mm^{-1}$ for analytical work; 7.8 mm × 30 cm, flow 5 $ml \cdot mm^{-1}$ for preparative work).

Differential scanning calorimetry (DSC) measurements. Calorimetric measurements were performed on the dry and on the hydrated sample, using a differential scanning calorimeter (Mettler TA 3000). Sealed capsules were employed to prevent adsorption of water during the measurements on the dry sample. Measurements on the hydrated sample were performed in the presence of excess water, to ensure complete hydration. The warming or cooling rate was usually 5 C deg./min. Determination of the transition points and of the enthalpy production was performed on line, by means of a microprocessor connected to the calorimeter.

Capacitance and conductance measurements on black films. Black membranes were formed on a circular hole (0.8 or 0.6 mm in diameter in different experiments) from a 25 mg/ml dispersion of asymmetric lipid in squalene (Sigma Chemical Co., St. Louis, MO). The best temperature value at which membranes could be formed was just below the higher transition value. No formation at all occurred below the lower transition temperature.

Membrane capacitance was measured at 500 Hz (V_{AC}(max) = 40 mV) with a precision (0.1%) a.c. impedance bridge. Ag | AgCl electrodes were employed for applying and recording the signal. A voltage clamp circuit, based on a LF 356 (National Semiconductor) operational amplifier was used to apply the signal. Membrane conductance was determined using a square wave of 20 mV peak-to-peak at 0.1 Hz. A current voltage converter, based on an operational amplifier (LH 0052 National Semiconductor) was employed to monitor the signal on an Hp 7402 oscillographic recorder. Valinomycin (Calbiochem, Los Angeles, CA) was added to the aqueous phase from ethanolic stock solution ($3 \cdot 10^{-3}$ M). The sensitivity of conductance measurements was around 5%. The area of the membrane was determined photographically using transmitted light. The estimated error was of the order of 0.5%. Most of the capacitance-temperature curves were performed on the same membrane by varying the temperature at a rate of 1 C deg./min. However, in some cases (e.g., for the 4-asymmetrical lipid above 60°C), owing to film instability, measurements had to be performed on different samples. To obtain significant data, the error in the area had to be reduced to 0.3% and this was achieved by weighing a photographic magnification (280 ×) of the membrane itself. In both cases, the experimental error in capacitance did not exceed 1%. Ohter experimental details can be found elsewhere [15,17].

Results

Differential scanning calorimetric thermograms, performed on dry samples of the crude mixture of the symmetrical and asymmetrical molecule and of the three separated asymmetrical compounds, are shown in Fig. 2. They clearly reveal the existence of two endothermic transitions in the asymmetrical molecule which are shifted towards higher temperature values as the number of cycles increases. The enthalpy production, ΔH, the corresponding entropy variation, ΔS, and the peak temperatures, t_i, are collected in Table I. Data pertain to the warming mode; they are the mean value over four different measurements. A hysteresis of up to 5 C deg. can be observed in the cooling mode. Single measurements of ΔH can be affected by deviations of up to 20%, due to the broadness of the peak (which in turn may depend on the scanning rate). Because of this behaviour, there is a certain arbitrariness in the choice of the base-line in the peak integration procedure. The peak temperature rarely varied by more than 2°C in different DSC measurements.

Notice that a completely different kind of transition is observed in the symmetrical molecule. In this case, a unique peak with a 10-fold higher enthalpy production was detected. DSC measurements performed in the presence of excess water (to assure complete hydration) simply revealed a broader peak with a 2°C higher transition temperature (26.9°C) and a 30% smaller ΔH (13 J/g), as Fig. 3a and b show. By contrast, great differences in the thermograms of the hydrated asymmetrical molecule, with respect to the dry one, were observed, as shown in Fig. 3c,d. Besides other significant differences, the hydrated compound showed a new peak, similar to that detected for the symmetrical molecule, peaking around 26°C, with a $\Delta H =$ 6 J/g. The higher temperature transitions were becoming smaller and nearer. Furthermore, a change in slope was observed at the highest transition temperature. When the sample was again dried, the usual DSC thermogram appeared, as shown in Fig. 3d.

Fig. 4a and b show the specific capacitance-temperature curve of 4- and 5-asymmetric lipid black membranes, formed on a circular hole 0.8 mm in diameter. To bring out the trend more

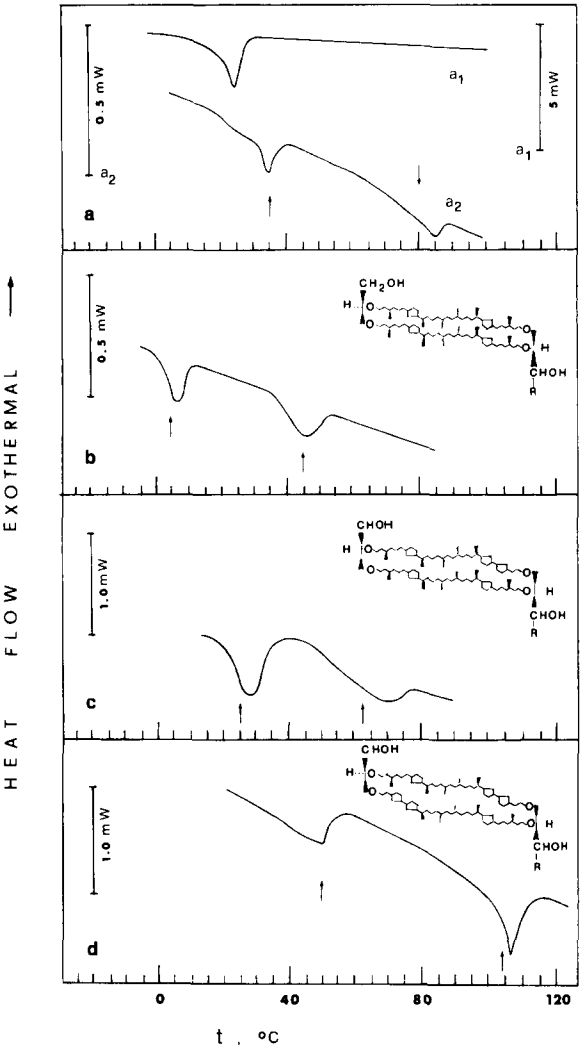
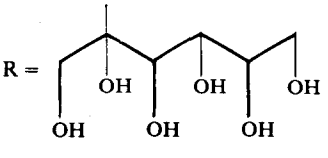


Fig. 2. Differential scanning calorimetric thermograms of lipid dry samples, performed with a scan rate of 5 C deg./min: (a_1) Symmetric lipid native mixture (referred to the right-hand scale); (a_2) Asymmetric lipid native mixture (referred to the left-hand scale); (b) 4-asymmetric lipid; (c) 5-asymmetric lipid; (d) 6-asymmetric lipid. The arrows indicate the peak temperatures corrected for the parasite thermal effects. The insets indicate the structure of lipid molecules with different degrees of cyclization, where



clearly, results were not mediated from different experiments. However, the behaviour on different

TABLE I

The transition points, t_i, the apparent enthalpy productions, ΔH_i, and the corresponding entropy variation, ΔS_i, as deduced by DSC measurements, are given for the indicated lipid dry samples.

Lipid	t_1 (°C)	ΔH_1 (J/g)	ΔS_1 ($\times 10^3$) (J/g per K)	t_2 (°C)	ΔH_2 (J/g)	ΔS_2 ($\times 10^3$) (J/g per K)
4-Glycerol-dialkyl-nonitol	5	1.5	5.4	43	1.4	4.4
5-Glycerol-dialkyl-nonitol	25	2.8	9.4	60	2.4	7.2
6-Glycerol-dialkyl-nonitol	48	0.33	1	104	1.1	2.9
Glycerol-dialkyl-nonitol (native mixture)	36	1.8	5.8	84	2.1	6.4
Glycerol-dialkyl-glycerol (native mixture)	24.6	19.8	66.5	–	–	–

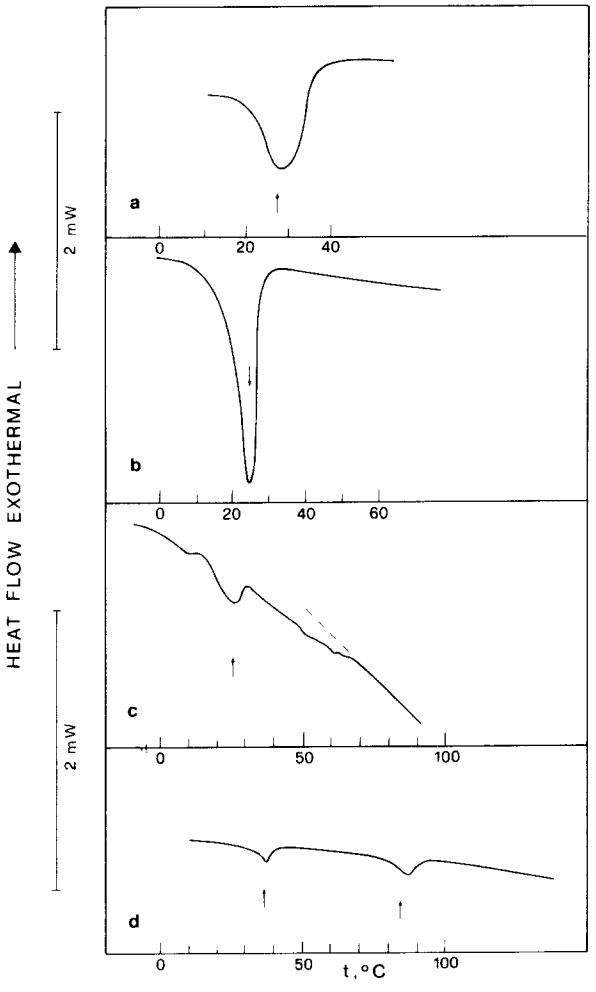


Fig. 3. Differential scanning calorimetric thermograms of the lipid crude mixture. (a) Hydrated symmetrical; (b) dry symmetrical; (c) hydrated-asymmetrical; (d) dry asymmetrical lipid mixture. Thermograms (b) and (d) are obtained after complete removal of water and are reported for comparison. The arrows indicate the peak temperature corrected for the parasite thermal effects.

membranes is qualitatively identical. To get a full cooling-warming cycle we had to prevent the breakage of the membrane usually observed below 20°C. Therefore, membranes were often formed around 60°C and rapidly cooled to 30°C, in order to start measurements from this temperature value. The experiment shown in Fig. 4b is performed with such a precaution.

Common feature to both curves is a bell-shaped configuration and a marked hysteresis, already observed for an asymmetric lipid crude mixture in various solvent systems [18]. As expected, the maximum capacitance value and the change in sign of dC_s/dt occur at temperatures which are peculiar to any molecular species.

Information on membrane structural changes, induced by lowering the temperature, could also be obtained by adding valinomycin to the aqueous solution and measuring the membrane conductance. Fig. 4c shows the result. The straight line indicates exponential behaviour of conductance as a function of $1/T$. The corresponding activation energies are $\Delta G_1 = 14.2$ kcal/mol for $t < 37$°C and $\Delta G_2 = 9.6$ kcal/mol for $t > 37$°C. The breaks in conductance occur near the two temperatures at which the abrupt changes of dC_s/dt occur. Similar values of activation energies were found for a crude mixture of asymmetric lipid in squalene [18] and also for a normal bilayer of dieurucoylphosphatidylcholine [19].

The behaviour of capacitance shown in Fig. 4a and b induced us to look for the occurrence of a third structural transition, at higher temperatures, in analogy with the calorimetric findings on the hydrated compound. However, the membranes

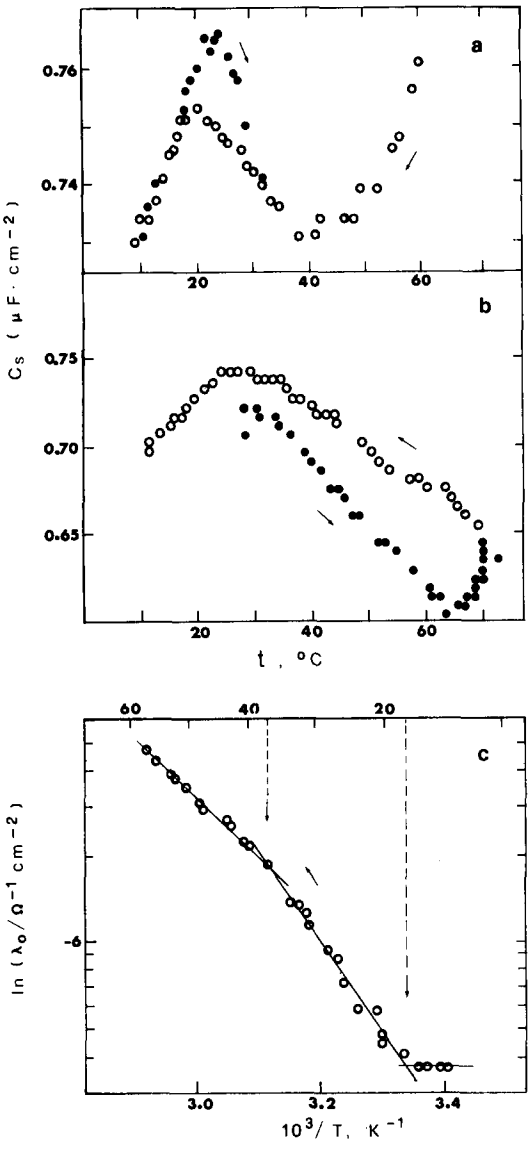


Fig. 4. Specific capacitance (a,b) and conductance (c) measurement on black membranes from squalene dispersion of: (a) 4-asymmetric lipid; (b,c) 5-asymmetric lipid. Measurement (c) is performed in the presence of $5 \cdot 10^{-8}$ M valinomycin in the external solution. The arrows underline the correspondence between the major changes in the slope of $C_s(t)$ and the break in the logarithm of conductance. Ionic solution: 0.1 M KCl. ●, pattern of increasing temperature; ○ pattern of decreasing temperature.

were quite unstable at $t > 60°C$; to improve their stability, they were formed on a smaller hole (0.6 mm in diameter). Even in such a case, we did not succeed in performing an entire cooling cycle on

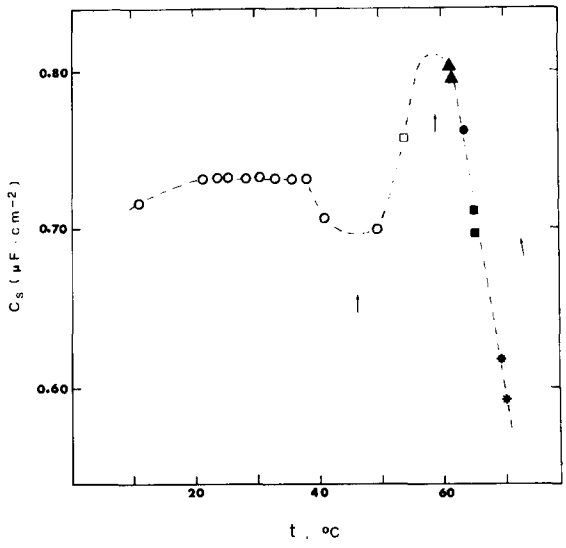


Fig. 5. Specific capacitance of a black membrane from a 4-asymmetric lipid dispersion in squalene. The different symbols show measurements performed on different membranes. The lower temperature values pertain to a pattern of decreasing temperature. Notice the big change in capacitance near the two higher transition temperatures (indicated by the arrows) and the flattening of the lower temperature peak, possibly due to the smaller dimensions of the hole. Ionic solution: 0.01 M KCl.

the same membrane. Therefore the results shown in Fig. 5 pertain to several membranes whose area was carefully measured as described in Materials and Methods. The trend of the curve indicates that a third transition does indeed occur at higher temperature, while the two lower temperature transitions are similar to those of Fig. 4a, except for a flattening. This difference in shape might be due [20] to the different dimensions of the hole, resulting in an area ratio of about 2 for the black membranes.

We could not perform capacitance measurements on the 6-asymmetric lipid during a cooling-warming cycle, since membranes were not forming below 70°C, while just above such a temperature they were very short-lived films. Their capacitance was 0.774 ± 0.009 μF/cm^2.

Discussion

The main problem posed by the present set of data concerns the nature of the thermal transitions exhibited by the system. Let us first consider the

calorimetric measurements performed on the dry sample. The most informative finding is the completely different behaviour of the thermal transition in the case of the symmetrical and the asymmetrical molecule. Since the two molecules differ only in the polyvalent alcohol, nonitol, the latter finding suggests that the two thermal transitions are related to the presence of the nonitol-containing polar head. In its fully stretched configuration, nonitol is about 10 Å in length, while it does not exceed 60 $Å^2$ in cross-sectional area in its most compact configuration. The cross-sectional area of the 4-asymmetric lipid molecule, calculated via its Van der Waals radii [21], is 92 $Å^2$ and this increases by about 7 $Å^2$ for the 6-asymmetric compound. Thus, the molecular packing of the lipid is determined by the hydrophobic interactions between the alkyl chains, but the presence of nonitol in one polar headgroup may establish dipolar interactions through the formation of hydrogen bonds. We suggest that the two thermal transitions observed on the dry asymmetric compound are due to the formation (during the cooling mode) or breakage (during the warming mode) of such hydrogen bonds at critical temperature values. The enthalpy production given in Table I seems to be somewhat low for such a process; for example, for the 5-asymmetric lipid (whose molecular weight is 1470), it is 900 cal/mol for both transition points, while a hydrogen bond requires at least 3 kcal/mol. However, one should consider that the process involves hydrogen bonds between two different polar heads which cannot freely interact with each other, owing to the peculiar structure and eventually to the low mobility of the polar head groups in the dry system. Consequently, only a fraction of the total number of molecules is expected to establish intermolecular bonds through their polar head groups.

Data of Fig. 2 and Table I show that the transition peaks are shifted towards temperatures which are higher, the greater the number of cycles of the molecule. This behaviour is in agreement with the usual finding that two different compounds, having similar chemical behaviour (e.g., two noble gas or two isotopes), undergo a transition at a temperature ratio which increases with the ratio of their inertial moments. In the present case, an important role is probably also played by the different degree of freedom in bending and rotating of the nonitol-containing polar headgroup. In fact, it should be observed that the insertion of a ring in 5-asymmetric lipid occurs on the side of the nonitol-containing polar head and that the added cycle has a position, both in the 5- and the 6-asymmetric lipid, very close to the polar head itself. These considerations indicate that, even in the dry sample, the molecules are in a viscous-like state.

Calorimetric measurements on symmetrical lipid samples indicate the presence of a phase transition which has the characteristics of melting. Such a transition is present in the dry as well as in the hydrated state. By contrast, in the case of the asymmetrical compound, the phase transition, over the observed temperature range, is hindered, in the dry sample, by the existence of intermolecular hydrogen bonds, while it is partially allowed in the hydrated sample owing to the formation of a looser structure. In fact, the presence of water may change the number and decrease the strength of the hydrogen bonds formed in a pure lipid structure. Notice that the enthalpy of this transition is almost half of that produced by the symmetrical compound (6 J/g against 13 J/g). This seems to indicate that, because of the constraints between the polar headgroups, only a partial melting of the hydrophobic chains occurs. To understand better the rôle of water in such a transition, measurements are being repeated on the separated hydrated lipid molecules with and without the nonitol polar head.

The data presented in Figs. 4 and 5 indicate that bipolar lipid membranes formed from the asymmetric lipid at two different degrees of cyclization also undergo significant structural changes with temperature [18,22,23]. The transition points are shifted towards higher values with respect to the dry system in the 4- and the 5-asymmetric lipid. This is not surprising, considering the quite different topological arrangement of the molecules in the membrane and the presence of water, presumably bound water, near the polar headgroups.

The present set of data seems to indicate that the melting-freezing transition of the hydrophobic core ,revealed by calorimetric measurements only in the hydrated sample, occurs also in black films of asymmetric lipid molecules. Let us consider a

cooling run: the falling part of the capacitance curve, shown in Fig. 4a,b and Fig. 5, might be related to an increase in the membrane thickness, due to a liquid crystal-gel phase transition [18,23,24]. The lower temperature break, observed in the conductance logarithm of the 5-asymmetric sample, in the presence of valinomycin (Fig. 4c), occurs at about the same temperature (approx. 20°C) at which a change in sign of the capacitance slope occurs. Upon further lowering the temperature, the conductance does not vary any more, as expected for a frozen membrane in which valinomycin is almost immobilized. Moreover, most of the membranes were breaking at lower temperatures ($t < 17$°C), as often observed in usual lipid bilayers near the liquid crystal-gel transition point.

To obtain a complete observation of the capacitance-temperature behaviour, let us consider Fig. 4 where, owing to the lower transition temperatures of the 4-asymmetric sample, capacitance measurements do include both the transitions at the higher temperature. The latter induce variations in capacitance of up to 30%. Such a change cannot be simply explained in terms of variations in thickness. In fact, if only the thickness of the membrane varied, assuming the dielectric constant $\varepsilon_r = 2.1$, there would be a change in thickness of the membrane of 8 Å, from 31 to 23 Å. Such a variation in thickness seems to be too high in an almost solvent-free membrane lacking a midplane region [17]. Furthermore, a thickness of 23 Å seems to be too low for an alkyl chain, 32 carbon atoms long, whose stretched length is at least 40 Å. An alternative, and more convincing, hypothesis in line with the previous interpretation of calorimetric measurements, on dry samples, is one which the major rôle in capacitance variations is played by changes in the mean value of the dielectric constant. The latter would be induced by changes in dipole moments of hydrogen bonds, near the transition points. Bound water and ion-dipole interactions may also play a rôle in such behaviour. The present system thus reveals appealing analogies with a crystal in which a ferroelectric transition is induced by the formation of hydrogen bonds [25]. Preliminary experimental work is in agreement with such an expectation and theoretical work is in progress along these lines.

In conclusion, these measurements have given indications of one of the physiological roles of cyclization in the plasma membrane of *C. acidophila*. In fact, we have shown that the degree of cyclization acts as a buffer against external temperature variations by determining the temperature at which the transition points, due to the polar heads, occur. An additional rôle, which is not directly investigated by the present study, may be that of modulating the microviscosity and the melting point of the hydrophobic core.

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