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Tetraether Lipid Components from a Thermoacidophilic Archaebacterium

Chemical Structure and Physical Polymorphism

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As a continuation of an X-ray scattering study of the tetraether lipids extracted from the thermophilic archaebacterium *Sulfolobus solfataricus*, the phase behaviour of four fractions of the complex polar lipid extract (PLE) is described. Each molecule of two of these fractions (P1 and GL) carries an unsubstituted glycerol headgroup, those of another (P2) no such group; the fourth fraction (WPLE) is obtained by water-washing PLE, thus reducing its P2 content from approximately 48% to approximately 24% and increasing the average number of molecules bearing an unsubstituted glycerol headgroup from approximately 0·4 to approximately 0·6. The main result is a striking correlation between the phase behaviour and the average ratio of unsubstituted glycerol headgroups to the total number of headgroups: the fractions P1, GL and WPLE, in which that number is respectively 0·5, 0·5 and 0·3, form rod-containing phases; the fraction P2, in which that number is zero, yields a lamellar phase throughout the phase diagram. An analysis of the dimensions of the structure elements confirms our previous conclusion that, in the presence of a sufficient amount of water, the unsubstituted glycerol headgroups partition preferentially in the hydrocarbon regions rather than at the polar/apolar interfaces. These results, moreover, corroborate our previous conjectures regarding the correlations between the structure of the plasma membrane, the phase behaviour of the lipid extract and life at high temperature.

1. Introduction

Archaebacteria are a novel class of micro-organisms that, over the last few years, have bloomed into a very active field of research and raised a variety of fascinating problems (for a review, see Woese & Wolfe, 1985). One of these is the peculiar chemical structure of the lipids, sharply different from that of the lipids of eubacteria and eukaryotes: the hydrocarbon moiety consists of isoprenoid chains (possibly containing some cyclopentanes); the hydrocarbon chains are ether (instead of ester)-linked to the headgroups; the glycerol stereochemistry is 2,3-di-*O*-*sn*-glycerol (instead of *sn*-1,2) (for a review, see Langworthy & Pond, 1986; Luzzati *et al.*, 1987*a*). Our long-standing interest in lipid polymorphism drew our attention to those lipids, and more particularly those that consist of a pair of C_{40} hydrocarbon chains, each ether-linked at each end to a polar headgroup (DeRosa & Gambacorta, 1986). These most unusual tetraether molecules are the major

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lipid component of several extremely thermophilic organisms.

We have reported (Gulik *et al.*, 1985) an X-ray scattering study of several lipid–water systems containing the polar lipid extract (PLE)† and two hydrolytic derivatives (called GDGT and GDNT) of *Sulfolobus solfataricus*, a thermophilic archaebacterium whose lipid molecules are virtually all (>95%) tetraethers. This work led to a variety of observations and conclusions, some of which seemed to be of particular interest:

(1) a high fraction of the tetraether lipid molecules contain one unsubstituted glycerol headgroup: this type of lipid headgroup is totally absent among "ordinary" lipids, as well as among diether archaebacteria lipids;

(2) the unsubstituted glycerol headgroups partition preferentially in the hydrocarbon regions, rather than at the polar/apolar interfaces;

(3) cubic phases are observed at temperature and water content close to physiological;

(4) the polymorphic transitions involving the cubic phases display a most unusual metastability.

The hydrolytic derivatives GDNT and GDGT used in our previous work bear respectively one and two unsubstituted glycerol headgroups (see chemical composition below): we call these two types of molecules monosubstituted and zerosubstituted, respectively. The PLE is a mixture of lipid molecules some of which (P1, GL, SL, see below) are monosubstituted and some (P2) bisubstituted (i.e. the molecules bear no unsubstituted glycerol headgroup). We studied in this work three lipid fractions of PLE (two (P1, GL) are monosubstituted and one (P2) bisubstituted) and also P2-impoverished PLE (WPLE); our purpose is to reinforce some of the conclusions drawn from the study of PLE, GDGT and GDNT and to verify our inferences regarding biological significance (Luzzati & Gulik, 1986; Luzzati *et al.*, 1987*a*).

2. Materials and Methods

The micro-organism *S. solfataricus* strain MT-4 was grown at 85°C and pH 3·5. The fraction PLE was obtained from dried cells by extraction with chloroform/methanol (1:1, v/v) followed by washing with *n*-hexane. WPLE was obtained by extensive washing of PLE with water. The separation of the constituents of PLE was achieved by chromatography on a silica gel column. Elution with chloroform/methanol (97:3, v/v), chloroform/methanol/water (65:25:1, by vol.), chloroform/methanol/water (65:25:4, by vol.), methanol/water (1:1, v/v) yielded respectively the fractions GL, a mixture of P1 and SL, a mixture of trehalose (synthesized by the bacteria) and P2, and finally trehalose with a trace amount of P2. P1 was separated from SL by flash chromatography. P2 was separated from trehalose by washing with water. For the chemical composition of the different fractions, see below and Fig. 1.

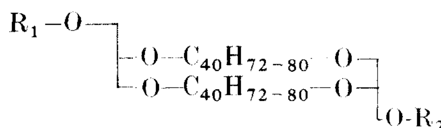
The phase diagrams were explored using lipid–water samples of known composition. The X-ray scattering experiments were performed and analysed as described by Gulik *et al.* (1985). In the lamellar phases, d_{par} is the partial thickness of the hydrocarbon layer; in the hexagonal and the cubic phases R is the radius of a polar rod; in all the phases S_{ch} is the area/hydrocarbon chain at the polar/apolar interface.

† Abbreviation used: PLE, polar lipid extract.

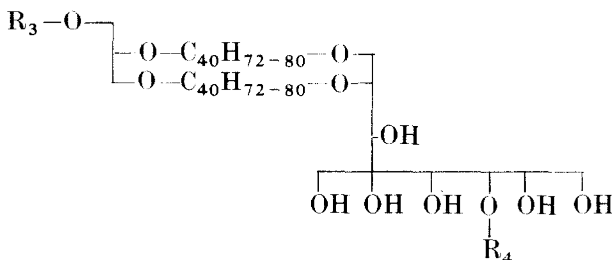
3. Chemical Composition

All the lipid molecules contain two biphytanyl chains ether-linked either to two glycerol or to one glycerol and one nonitol headgroup; a variety of polar groups are linked to the glycerol and the nonitol groups. Each biphytanyl chain contains up to four cyclopentane rings. All the molecules derive from the two compounds GDGT and GDNT (DeRosa *et al.*, 1983), the chemical structures of which are sketched in Figure 1.

GDGT with $R_1 = H$, $R_2 = H$



GDNT with $R_3 = H$, $R_4 = H$



P1 is a derivative of GDGT, with $R_1 = H$, R_2 = phosphomyoinositol.

GL is a mixture of: 70% derivative of GDNT, with $R_3 = H$, R_4 = β-D-glucopyranose; 30% derivative of GDGT, with $R_1 = H$, R_2 = β-D-galactopyranosyl-β-D-galactopyranose.

P2 is a mixture of: about 90% derivative of GDNT, with R_3 = phosphomyoinositol, R_4 = β-D-glucopyranose; about 10% derivative of GDGT with R_1 = phosphomyoinositol, R_2 = β-D-galactopyranosyl-β-D-glucopyranose.

SL is a derivative of GDNT, with $R_3 = H$, R_4 = β-D-glucopyranosyl sulphate.

PLE is the polar lipid extract: it contains 6% P1, 15% GL, 48% P2, 8% SL.

WPLE is water-washed PLE, with the P2 content reduced from approximately 48% to approximately 24%.

4. Results

We describe below the results obtained with the lipid fractions PL, GL, P2 and WPLE, whose chemi-

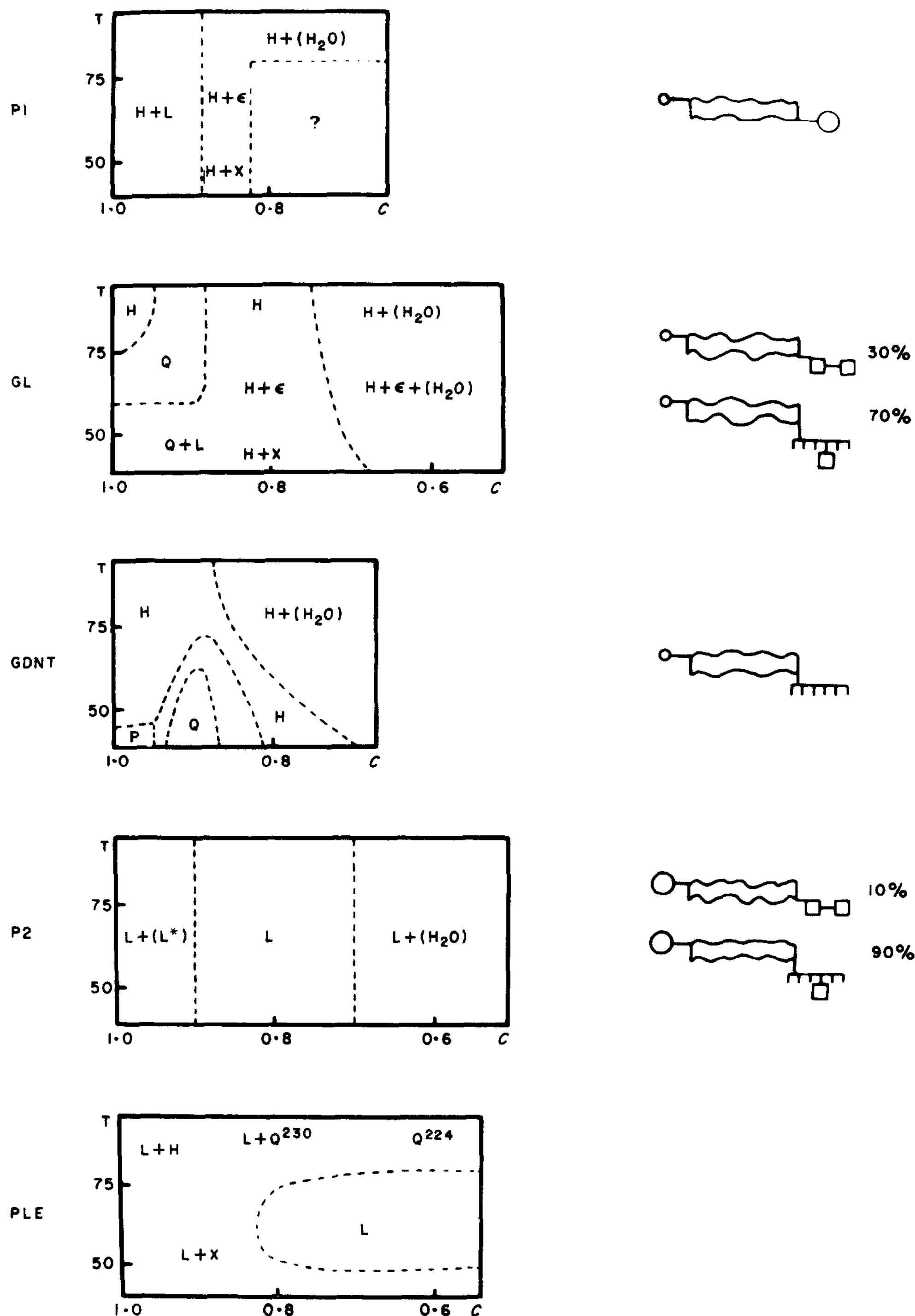


Figure 1. *Left:* representation of the phase diagrams of the lipid–water systems studied in this work (those of GDNT and PLE are reproduced from Gulik *et al.*, 1985). Note that the multiphase regions were not carefully explored, nor were the position of the phase boundaries accurately determined (see the text). The phase notation is: L, lamellar, with 1 lipid lamella/motif (structures II and V in Figs 2 and 3); L*, lamellar, with 2 lipids lamellae/motif (structures IV and VI); H, hexagonal (structure I); Q, cubic (Q^{230} unless otherwise specified); P, rectangular (structure III). X indicates the presence of unidentified sharp reflections,? the presence of unidentified diffuse and sharp reflections, ε the presence of trace amounts of unidentified phase(s). *Right:* schematic chemical structure of the lipid fractions. *Wriggles*, hydrocarbon chains. *Small circles*, unsubstituted glycerol OH. *Large circles*, phosphomyoinositol. *Square*, sugar residue. *Comb*, nonitol.

cal composition is given above. The results relevant to GDNT, GDGT and PLE have been reported elsewhere (Gulik *et al.*, 1985). The phase diagrams were explored using samples of variable lipid–water composition, each at variable temperature. Several phases were identified: the small amounts of lipids available hindered a precise location of the phase boundaries. Moreover, as it often the case with

lipid–water systems, the phase behaviour is sometimes unclear at low temperature and low water content.

We are concerned here only with the phases in which the conformation of the hydrocarbon chains is disordered (type α). The hexagonal and cubic phases observed in this work are of type II: all consist of polar rods embedded in a hydrocarbon matrix (for a description of the phases, see Gulik *et al.*, 1985).

For the lipid fractions containing unsubstituted glycerol headgroups, the determination of the dimensions of the structure elements depends upon the partition of the unsubstituted glycerol headgroups between the hydrocarbon regions and the polar/apolar interface. Previously (Gulik *et al.*, 1985), we considered two extreme cases:

Type A: the unsubstituted glycerol headgroups segregate away from the polar/apolar interface into the hydrocarbon matrix

Type B: the unsubstituted glycerol headgroups are located at the polar/apolar interface.

The choice between type A and B was based upon an analysis of the dimensions of the structure elements, more specifically of the variation of S_{ch} as a function of water content and temperature, and involved the notion that S_{ch} is bound to increase with increasing temperature and water content, and that its value cannot be smaller than 20 $Å^2$.

(a) *P1*

This lipid fraction consists of monosubstituted molecules. Because of the tiny amount of lipid available, the analysis of this phase diagram is particularly scanty.

At high temperature and high water content ($t > 75$°C, $c < 0{\cdot}9$) only the hexagonal phase is observed (Fig. 1); the parameters relevant to a few experiments are reported in Table 1. Using the same arguments as those involved in the analysis of the phase H of GDNT (Gulik *et al.*, 1985), it is possible to conclude that the partition of the unsubstituted glycerol headgroups is of type A (structure I in Fig. 2).

For $c > 0{\cdot}9$, the phase diagram is more complex. In the dry sample two phases are observed. One, present at all temperatures, seems to be lamellar (although its reflections are weak and not very sharp), with a repeat $a = 84$ Å; the unit cell most

Table 1

Dimensions of the structure elements

P1

c	1			0·86		0·81	
t	50			90		90	
Phase	L*	H		H		H	
a (Å)	83·9	50·8		71·6		79·7	
Case	B	A	B	A	B	A	B
d_{par} (Å)	69						
R (Å)	–	10	11	19·3	20·3	23·3	24·4
S_{ch} ($Å^2$)	29·2	34	19	38·9	20·5	40·6	21·3
Figure	IV	I		I		I	
	↑	↑		↑		↑	

GL

c	1		1		0·93		0·80		0·70	
t (°C)	68		90		90		90		60	
Phase	Q^{230}		H		Q^{230}		H		H	
a (Å)	120·5		57		148·5		87·5		102·3	
Case	A	B	A	B	A	B	A	B	A	B
R (Å)	10·4	11·5	12·5	13·7	15·2	16·1	27·1	28·2	35·4	36·3
S_{ch} ($Å^2$)	39·8	21·4	35·6	19·6	39·7	20·8	41·5	21·5	45·6	23·4
Structure	—	—	I	—	—		I		I	
			↑				↑		↑	

P2

c	1	0·92	0·78
t (°C)	55	69	91
Phase	L	L	L
a (Å)	49·3	52·6	55·9
d_{par} (Å)	35·7	34·7	30·7
S_{ch} ($Å^2$)	28·3	29·1	32·9
Structure	V	V	V
	↑	↑	↑

A few examples of well-defined phases (see Fig. 1). c, weight concentration (lipid/(lipid + water)); t, temperature; H, L, Q^{230}, nature of the phases; d_{par}, partial thickness of the hydrocarbon layer; R, radius of the polar rods; S_{ch}, area/chain at the polar/apolar interface. The values of d_{par}, R and S_{ch} are calculated for the 2 cases A and B (see the text and Gulik *et al.*, 1985). Reference is given to the structures sketched in Figs 2 and 3. The case chosen (see the text) is marked by an arrow.

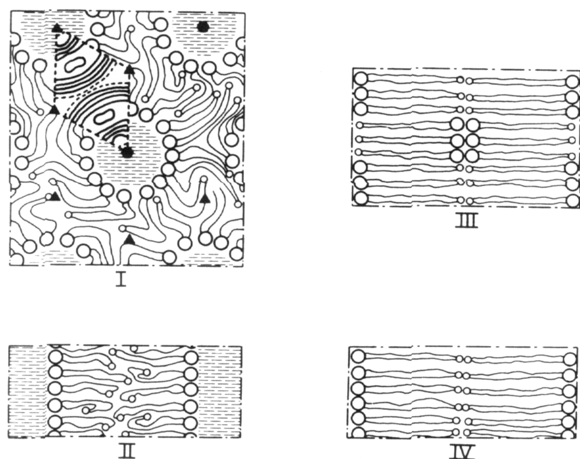


Figure 2. Representation of the structure of the phases observed with the monosubstituted molecules. *Wriggles*, hydrocarbon chains. *Small circles*, unsubstituted glycerol headgroups. *Large circles*, substituted glycerols and substituted or unsubstituted nonitol headgroups. The hatched areas represent the water. *Left:* highly hydrated samples, with the unsubstituted glycerols embedded in the hydrocarbon matrix. I, hexagonal phase (H), observed in GDNT, P1, GL and WPLE (inset: electron density distribution). II, one type of lamellar element of the phase Lα of PLE. *Right:* little hydrated or dry samples, with the unsubstituted glycerol headgroups segregated away from the other types of headgroups, and sitting at the surface of the lamellae. III, rectangular phase P; IV, lamellar phase L* with a pair of lipid lamellae/motif.

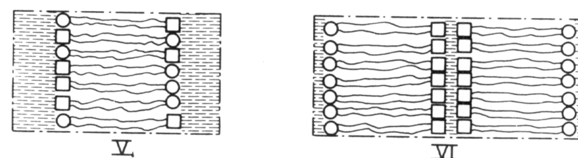


Figure 3. Representation of the structures of the phases observed with the disubstituted molecules of P2. Notation as above, with *large circles* and *squares* representing respectively the phosphomyoinositol and the sugar-containing residues. V, lamellar structure, with 1 lamella/motif; the lipid molecules, randomly oriented, span the lipid layer, VI, lamellar structure, with 2 lamellae/motif; the 2 types of polar headgroups segregate from each other; the lipid molecules span the lipid layers.

likely contains two lipid lamellae, as sketched in IV (Fig. 2). At low temperature, the second phase seems to be the rectangular P (III, Fig. 2) previously reported in GDNT; above 50°C this phase transforms into a hexagonal phase of type A (see Table 1). In all these structures the two types of headgroups segregate away from each other. Besides, as P1 is a pure chemical species, save for the heterogeneity of the hydrocarbon chains, the presence of two phases in the absence of water is surprising, and may be explained either by the presence of some impurity (as hinted by thin-layer chromatography) and/or by a heterogeneous ionization of the phosphates.

(b) *GL*

This lipid fraction is also monosubstituted. At $t=90$°C a hexagonal phase is found, pure, both at low water content and at $c<0{\cdot}85$. The cubic phase Q^{230} is found, pure, at $c=0{\cdot}93$, $t=90$°C and at $c=1$, $t=68$°C. This phase behaviour (phase H deeply indented by phase Q, low-c-boundary of phase H strongly concentration-dependent) is similar to that of GDNT (see Fig. 2). The dimensions of the structure elements (see Table 1) show that, as for GDNT and P1, the partition of the unsubstituted glycerol headgroups is of type A.

(c) *P2*

This is the only lipid fraction of this and of the previous paper whose molecules are devoid of unsubstituted glycerol headgroups. At $c<0{\cdot}90$ a lamellar phase is observed over an extended portion of the phase diagram; the dimensions of the structure elements of a few examples are reported in Table 1. Since the value of d_{par} is shorter than the fully extended length (37 Å) of the hydrocarbon moiety, the most likely structure is the one sketched in V (Fig. 3), each molecule spanning the lipid lamellae (we compare below this structure and that of the phase Lα of PLE).

At $c>0{\cdot}90$ additional weak reflections are observed, consistent with a lamellar structure whose repeat distance is almost twice the length of a lipid molecule; this observation can be explained by a segregation of the two types of polar headgroups as sketched in VI (Fig. 3).

(d) *WPLE*

This lipid fraction derives from PLE by depletion of P2, and thus by enrichment of unsubstituted glycerol headgroups. Above the "melting" temperature of the hydrocarbon chains, a hexagonal phase is observed throughout the phase diagram. The lattice dimension varies with the water content, from 61 Å at $c=1$ to 94 Å in excess water (and at 50°C). Moreover, and by analogy with GL and GDNT (see Fig. 1), in the presence of excess water, the dimensions of the hexagonal lattice decrease dramatically with increasing temperature. The structure is also of type A.

5. Discussion and Conclusions

According to their phase behaviour, especially in the high temperature ($t>60$°C) and high water ($c<0{\cdot}9$) regions, the various lipid fractions can be put into two main classes: one, to which belong P1, GL, GDNT, WPLE, displays only rod-containing phases (H and Q); the other, represented by P2, only the lamellar phase. The behaviour of PLE, which is a mixture of lipids of the two classes, is

hybrid, with both lamellae- and rod-containing phases. The correlation between the class to which the lipid belongs and the (average) ratio of unsubstituted to substituted glycerol headgroups per molecule is striking: 1 for P1, GL, GDNT; approximately 0·43 for WPLE; approximately 0·25 for PLE; 0 for P2. Moreover, relying upon the notion that the average area per chain at the polar/apolar interface (S_{ch}) is bound to be larger than 20 Å^2 and to increase as the water content increases, one can show that the unsubstituted glycerol headgroups partition preferentially in the hydrocarbon regions and that the polar/apolar interfaces are occupied preferentially by the substituted glycerol and the substituted and unsubstituted nonitol headgroups. The fact is also worth noting that the values of S_{ch} observed with P1, GL and P2 (see Table 1) are in good agreement with those reported with GDNT and PLE (see Fig. 5 and Table 2 of Gulik *et al.*, 1985).

At c close to 1 lamellae-containing phases are sometimes observed (III, IV, Fig. 2) with the unsubstituted glycerol headgroups located at the surface of the lamellae: at low water content, therefore, those headgroups may no longer partition preferentially in the hydrocarbon regions (for the properties of GDGT, see also Gulik *et al.*, 1985). Note, though, that the two types of headgroups always segregate away from each other.

All these observations corroborate our previous conclusions regarding the low polarity of the unsubstituted glycerol headgroups. It is worth stressing that the presence among membrane lipids of a large proportion of unsubstituted glycerol headgroups seems to be unique to tetraether lipids: biphytanyl glycerol is not present among diether isopranyl lipids of archaebacteria (Kushwaha *et al.*, 1982), mono- and diglycerides are not usual membrane constituents of eubacterial and prokaryotic cells (a small amount of diglycerides has in fact dramatic disrupting effects on lipid phases; Das & Rand, 1986). Monosubstituted tetraethers (P1, GL) thus seem to behave more like ordinary monopolar lipid molecules, save for their exceptional length (approximately 40 instead of approximately 20 Å), than like genuine bipolar lipids such as P2.

Only P2, of all the tetraether lipids studied so far, displays a lamellar phase whose structure is "plain", in the sense that the thickness of the lipid layer (d_{par} in Table 1) is consistent with the presence of lipid molecules spanning the lamellae (V, Fig. 3). By contrast the lamellar phase of PLE is peculiar: the observation that the average thickness of the lipid layer ($d_{par}=39$ Å) exceeds the fully extended length ($d_{par}=37$ Å) of the lipid molecules indeed led us to postulate a mosaic structure involving two type of lamellar elements (see Fig. 4 of Gulik *et al.*, 1985). One, containing the bisubstituted molecules, is similar to P2 (V, Fig. 3); the other, containing the monosubstituted molecules, is similar to a lipid bilayer, with the polar headgroups sitting at the interface and the unsubstituted glycerols embedded in the hydrocarbon matrix (II, Fig. 2). The striking differences between the two classes of lipids reinforce that model.

Another interesting observation is that the complex phase behaviour of PLE (presence of two cubic and one lamellar phase, unusual structure of the lamellar phase, metastability of the phase transitions involving the cubic phases (Gulik *et al.*, 1985)) seems to be closely related to its chemical composition: removing approximately 50% of P2 (this operation yields WPLE) suffices to replace the cubic and the lamellar phases by a hexagonal phase throughout the phase diagram. It appears, therefore, in keeping with our previous conclusions, that the bipolar lipid molecules (P2) play a crucial role in the structure (and metastability) of the cubic and the lamellar phases of PLE. Since, moreover, our conjectures regarding the biological significance of the phase behaviour of PLE (resistance to wide thermal fluctuations, potential catalytic role of the cubic phases, structure of the plasma membrane (Luzzati & Gulik, 1986; Luzzati *et al.*, 1987*a*)) were focused on the cubic phases, we are now led to believe that the chemical composition of *S. solfataricus* lipids is carefully balanced in the light of its functions.

One final comment is necessary regarding the biological significance of lipid polymorphism. Over the last 25 years, along with the exploration of the extraordinary polymorphism of lipid-containing systems, the hypothesis has been elaborated that this phenomenon may well play a physiological role: this hypothesis, though, has received scanty experimental support (for a review, see Gulik *et al.*, 1985). It is highly rewarding to note that the polymorphism of tetraether archaebacterial lipids (and also that of mono-olein, whose cubic phases seem to play a crucial role in the digestion of fats) brings the discussion much closer to the situation *in vivo* (Luzzati *et al.*, 1987*a*,*b*; Mariani *et al.*, unpublished results).

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