

## Stability and proton-permeability of liposomes composed of archaeal tetraether lipids

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### Abstract

Liposomes composed of tetraether lipids originating from the thermoacidophilic archaeon *Sulfolobus acidocaldarius* were analyzed for their stability and proton permeability from 20°C up to 80°C. At room temperature, these liposomes are considerably more stable and have a much lower proton permeability than liposomes composed of diester lipids originating from the mesophilic bacterium *Escherichia coli* or the thermophilic bacterium *Bacillus stearothermophilus*. With increasing temperature, the stability decreased and the proton permeability increased for all liposomes. Liposomes composed from tetraether lipids, however, remain the most stable. These data suggest these liposomes retain the rigidity of the cytoplasmic membrane of *S. acidocaldarius* needed to endure extreme environmental growth conditions.

**Key words:** Tetraether lipid; Liposome; Stability; Proton permeability; (Archaeon)

### 1. Introduction

The thermoacidophilic archaeon *Sulfolobus acidocaldarius* grows in extreme habitats of hot acid springs with pH values as low as 2 to 3 and temperatures of up to 85°C. The cell-envelope has a unique composition and protects the cells against these harsh environmental conditions. The cytoplasmic membrane is almost entirely composed of tetraether lipids that span the whole membrane. This monolayer organization is thought to provide the rigidity of the membrane needed under the extreme environmental conditions. The structure of tetraether lipids has been studied extensively [1–3]. These macro-cyclic membrane-spanning molecules consist of two C<sub>40</sub>-phytanyl chains linked through ether bonds to two glycerol molecules (Fig. 1). In contrast, membrane lipids of bacteria have their fatty acids bonded to glycerol through ester linkages. Some tetraethers have one of their glycerol molecules replaced by nonitol, a polyol with nine carbon atoms. The majority of the tetraether lipids are phosphoglycolipids containing galactose or glucose or both at one

side and *myo*-inositol phosphate at the other side of the molecule. In vivo, the sugar residues are at the outer face of the membrane, and thus are exposed to the low environmental pH [4]. Depending on the growth temperature, methyl side groups in the phytanyl chains can form up to four cyclopentane rings per chain [5] providing further rigidity to the membrane.

To understand the physicochemical basis for thermostability, much information has been gathered from the properties of dispersed individual tetraether lipid species. However, little is known about the features of the more complex mixture of lipids that constitutes the cytoplasmic membrane. A total lipid extract of *S. acidocaldarius* can be fractionated to yield a lipid fraction that forms closed unilamellar monolayer liposomes in aqueous media [6]. These lipids provide a suitable matrix for the functional reconstitution of membrane proteins that originate from bilayer-forming phospholipids. Both beefheart cytochrome *c* oxidase and the leucine transport system of *Lactococcus lactis* have been shown to be active after reconstitution in the tetraether lipids [6,7]. A recent comparison of the energy-transducing properties of primary transport systems reconstituted into liposomes of tetraether lipids and bacterial phospholipids suggested that the monolayer liposomes are endowed with a low proton perme-

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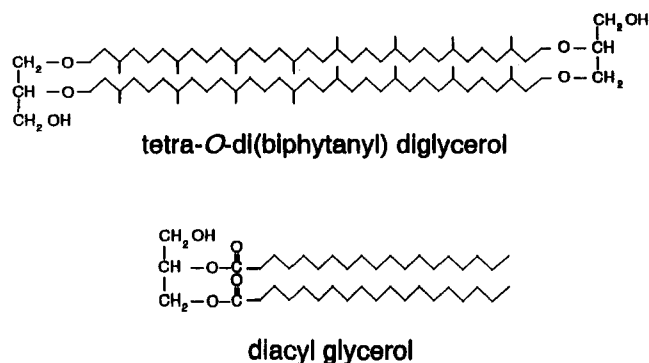


Fig. 1. Backbone of archaeal tetraether lipid and of bacterial phospholipid.

ability. Furthermore, many ionophores and protonophores are less potent when used in these liposomes, a characteristic that has been attributed to the rigidity of tetraether lipid membranes [8,9].

We now have analyzed the permeability properties of the monolayer liposomes for protons and small solutes like the fluorophore carboxyfluorescein. For comparison, liposomes were used prepared from bilayer-forming phospholipids extracted from the mesophilic bacterium *Escherichia coli* and the thermophile *Bacillus stearothermophilus*. The results demonstrate that tetraether liposomes have an exceptional low proton-permeability and long-term stability.

## 2. Materials and methods

### 2.1. Materials

5,6-Carboxyfluorescein (CF) was obtained from Eastman (Rochester, NY). Pyranine was obtained from Mol Probes (Eugene, OR).

### 2.2. Purification of lipids

*E. coli* phospholipid (*Escherichia coli* L- $\alpha$ -phosphatidylethanolamine type IX, Sigma) was purified by acetone-ether extraction as described [8]. Lipids were stored in chloroform under nitrogen at  $-20^{\circ}\text{C}$ .

*B. stearothermophilus* (ATCC 7954) cells were grown aerobically in a 10-l fermenter at  $60^{\circ}\text{C}$  in a medium containing 2% (w/v) tryptone, 1% (w/v) yeast extract and 1% (w/v) NaCl. Cells were harvested at an  $A_{660}$  of 1.5 to 2, collected by centrifugation and resuspended in 50 mM K-phosphate (pH 7). Cells were frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until use. Lipids were isolated according to Viitanen et al. [10] and stored in chloroform under nitrogen at  $-20^{\circ}\text{C}$ .

*S. acidocaldarius* (DSM 639) cells were grown aerobically in a 50-l fermenter at  $80^{\circ}\text{C}$  in Brock's medium supplemented with 5.8 mM L-glutamic acid, 50 mM  $\text{K}_2\text{SO}_4$  and 5.8 mM sucrose [11]. Cells were harvested

at an  $A_{546}$  of 1.5 to 2, concentrated by ultrafiltration using a Filtron Omega Centrasette membrane (Filtron Technology, Breda, the Netherlands) and freeze-dried. Freeze-dried cells of *S. acidocaldarius* (1.5 g) were soxhlet-extracted with 400 ml of chloroform/methanol (1:1, v/v) during 12 h. From the crude lipid extract a liposomal-forming fraction was isolated as described [8]. The lipids were suspended in chloroform/methanol/water (65:25:4, v/v/v) and stored at  $4^{\circ}\text{C}$  until use.

### 2.3. Sugar determination

The sugar content of *S. acidocaldarius* lipid was determined using the anthrone assay for hexoses [12].

### 2.4. Phosphate determination

The phosphate content of the different lipid preparations was determined with malachite green according to Driessen et al. [13] after destruction of dried lipid with 70%  $\text{HClO}_4$ .

### 2.5. Preparation of liposomes

*E. coli*, *B. stearothermophilus* and *S. acidocaldarius* lipids were dried by rotary evaporation and suspended in 50 mM potassium phosphate (pH 7.5), at a concentration of 15 or 20 mg/ml. Liposomes were obtained by sonication using a probe-type sonicator (intervals of 15 s sonication and 45 s rest) at  $0^{\circ}\text{C}$  under a constant stream of nitrogen. Sonication was continued until a clear suspension was obtained. Liposomes were stored in liquid nitrogen. Before use a small sample was slowly thawed at room temperature and extruded through 200 nm polycarbonate filters (Avestin, Ottawa, Canada), using a small volume extrusion apparatus (LiposoFast<sup>™</sup> Basic, Avestin) [14].

### 2.6. Particle sizing of liposomes

Size distributions of the different liposome preparations was determined by photon correlation spectroscopy using a 'Nicomp' submicron particle sizer (Avestin).

### 2.7. Electron microscopy

Cryo transmission electron microscopy was performed using a Philips CM 10 electron microscope with a liquid nitrogen-cooled gatan cryo device. Sample preparation involved liquid propane fast cooling.

### 2.8. Carboxyfluorescein release

Release of entrapped fluorescent dye 5,6-carboxyfluorescein (CF) was monitored according to Goessens

et al. [15]. Liposomes were loaded during sonication with 100 mM CF. After extrusion external CF was removed by chromatography of the liposomes over Sephadex G-25M columns. The concentration-dependent self-quenching of CF fluorescence permits continuous monitoring of leakage from liposomes. CF fluorescence was measured at excitation and emission wavelengths of 430 and 520 nm, respectively, in 50 mM K-phosphate (pH 7.5). The maximal level of CF fluorescence was measured after the addition of 0.1% Triton X-100.

## 2.9. Proton permeability

Proton permeability was measured in principle according to Nichols and Deamer [16]. Liposomes were loaded with 50 mM 4-morpholinepropanesulfonic acid (Mops) (pH 7), 75 mM KCl and 25 mM choline. After extrusion through 200 nm filters the external buffer was replaced by chromatography over Sephadex G-25 M columns PD-10 (Pharmacia, Uppsala, Sweden) in 0.5 mM Mops (pH 7), containing 75 mM KCl and 75 mM sucrose. Liposomes were diluted to 1.5 mg/ml in the external buffer and incubated with valinomycin (1 nmol/mg lipid). The fluorescent pH indicator dye pyranine (10  $\mu$ M) was added and the fluorescence was recorded at an excitation and emission wavelength of 450 and 508 nm, respectively. Fluorescence measurements were performed at increasing temperatures using a Perkin-Elmer LS-50 spectrophotofluorometer. A pulse of 100 nmol  $H^+$  (1  $\mu$ l 50 mM  $H_2SO_4$ ) was added and the backflow of  $H^+$  into the liposomes after the initial decay of the external pH was measured. Pyranine fluorescence was calibrated after each individual experiment by adjusting the external pH with  $H_2SO_4$  in the presence of nigericin (1 nmol/mg *E. coli* or *B. stearothermophilus* lipid and 50 nmol/mg *S. acidocal-*

*darius* lipid). Pyranine fluorescence was linear with the amount of  $H^+$  at the actual pH range. Upon addition of 100 nmol  $H^+$  the pH dropped about 0.2 pH units. The rate constant of  $H^+$  flow into the liposomes was calculated by curve-fitting of the exponential increase of the signal after the initial quick decay of the external pH. The curve fit equation used was:

$$f = a[1 - e^{-bx}] + c + dx$$

in which:  $a$  is the amplitude of exponential,  $b$ , the first-order rate constant,  $c$ , the zero intercept, and  $d$ , a drift parameter. This latter parameter was included in the computational analysis when data obtained at temperatures above 50°C were used, where a significant drift of the signal was evident.

## 3. Results

### 3.1. Composition and size-distribution of the liposomes

The properties of the tetraether liposomes prepared from *S. acidocaldarius* were compared with liposomes prepared from bilayer-forming phospholipids extracted from *E. coli* and the thermophile *B. stearothermophilus*. Fractionation of the crude lipid extract of *S. acidocaldarius* yields a fraction that is relatively enriched in phosphoglycolipids (see also Discussion). The natural complex lipid mixture contains at least 11 different lipid species [6]. The phosphate content of this mixture was 0.40  $\mu$ mol of inorganic phosphate per mg of dried lipid. With an average molecular mass of 1800, approximately 70% of the lipid species are phospholipids. The sugar content was 0.55  $\mu$ mol of glucose equivalents per mg of dried lipid. The amount of the lipids that must be glycolipids depends on the relative distribution of di- or monosaccharides. The backbone

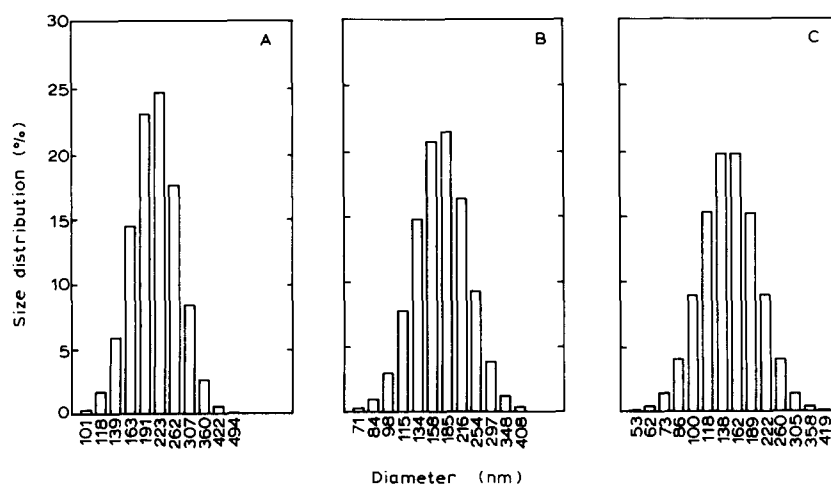


Fig. 2. Gaussian analysis of the size of liposomes composed of *S. acidocaldarius* lipid (A), *E. coli* phospholipid (B) and *B. stearothermophilus* phospholipid (C). The mean diameter of the liposomes was 219, 181 and 157 nm for A, B and C, respectively.

of these complex lipids is constituted by glycerol dialkyl glycerol tetraethers (GDGTs) as indicated in Fig. 1, or by glycerol dialkyl nonitol tetraethers (GDNTs) in which one of the glycerol molecules is replaced by nonitol. In vivo, the phosphate inositol is facing the cytoplasmic side of the membrane and the sugar residues and nonitols are facing the outside [4]. Liposomes must have lost this asymmetry in headgroup distribution as they carry a negative surface charge. This is evident from the fact that they are precipitated with the cationic polylysine, while addition of calcium elicited fusion among tetraether lipids (not shown). *E. coli* phospholipids used in this study contained approximately 65% phosphatidylethanolamine (PE), 30–35% phosphatidylglycerol (PG), and 0–5% cardiolipin (CL) [17]. The phospholipids extracted from *B. stearotheophilus* grown at a temperature of 65°C contained 21–32% PE, 22–39% PG and 23–42% CL [18].

From each lipid extract, unilamellar liposomes were prepared by extrusion through 200 nm polycarbonate filters. The size distribution of these liposomes was determined by photon correlation spectroscopy [19]. Liposomes of *S. acidocaldarius* lipids had a mean diameter of 219 nm with a standard deviation of the mean of 55 nm (Fig. 2A). The mean diameter of *E. coli* (Fig. 2B) and *B. stearotheophilus* (Fig. 2C) liposomes were  $181 \pm 52$  and  $157 \pm 48$  nm, respectively. Liposomes of *S. acidocaldarius* lipids were further analyzed by cryo electron microscopy (Fig. 3). This technique allows analysis of liposome shape without any mechanical deformation of the membranes. Most liposomes

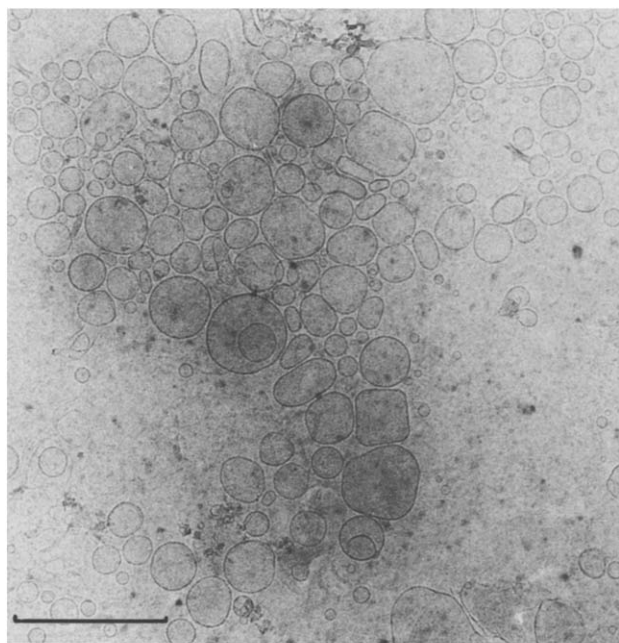


Fig. 3. Transmission electron micrograph of liposomes of *S. acidocaldarius* lipids after extrusion through 200 nm filters in frozen hydrated state. The bar denotes a distance of 0.5  $\mu$ m.

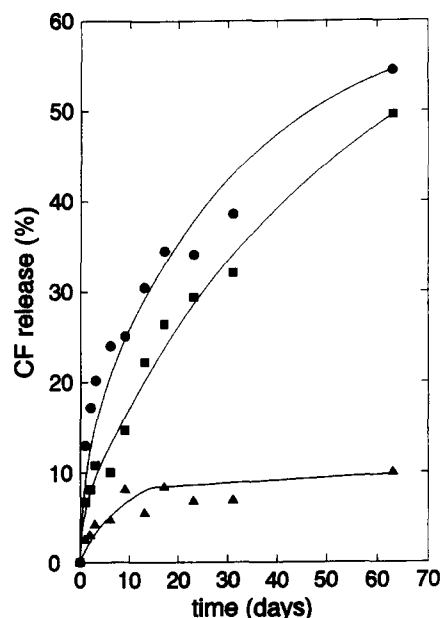


Fig. 4. Long-term stability of liposomes at room temperature. CF-loaded liposomes in 50 mM K-phosphate (pH 7.5) were incubated at room temperature in the presence of oxygen. The release of CF was measured during 62 days. The maximum fluorescence level (100%) was determined by the addition of 0.1% Triton X-100. Liposomes were composed of *S. acidocaldarius* lipids (▲ — ▲), *E. coli* lipids (■ — ■) and *B. stearotheophilus* lipids (● — ●).

appear to be unilamellar, although incidentally two concentric layers of liposomes are observed as indicated in Fig. 3. Extrusion of the liposomes effectively reduced the number of multilamellar liposomes to an insignificant level.

### 3.2. Thermostability of the liposomes

The relative stability of the liposomes was measured by following the time-dependent release of the fluorescent probe 5,6-carboxyfluorescein entrapped in the liposomal lumen. Release of CF is conveniently detected through the fluorescence increase which is due to a relief of self-quenching of the fluorophore present at a high concentration inside the liposomes. The different liposome preparations remained stable when stored at 0°C in the absence of oxygen. To investigate differences in stability, liposomes loaded with 100 mM CF were stored at room temperature in the absence of anti-oxidant and in the presence of oxygen. Moreover, liposomes were subjected to an osmotic shock by removal of external CF by chromatography over Sephadex, which led to an osmolarity of 427 and 111 mOsm on the in- and outside, respectively. Both liposomes composed of *E. coli* and *B. stearotheophilus* lipids gradually released about 50% of their CF contents over a period of 62 days (Fig. 4). Liposomes of *S. acidocaldarius* lipids, however, were much more stable. After an initial release of about 8–10% of CF during

the first 10 days, the remainder was retained in the liposomes over a long period of time. Similar experiments were conducted at higher temperatures, i.e., 50, 65 and 80°C during a period of 5 h (Fig. 5). At each

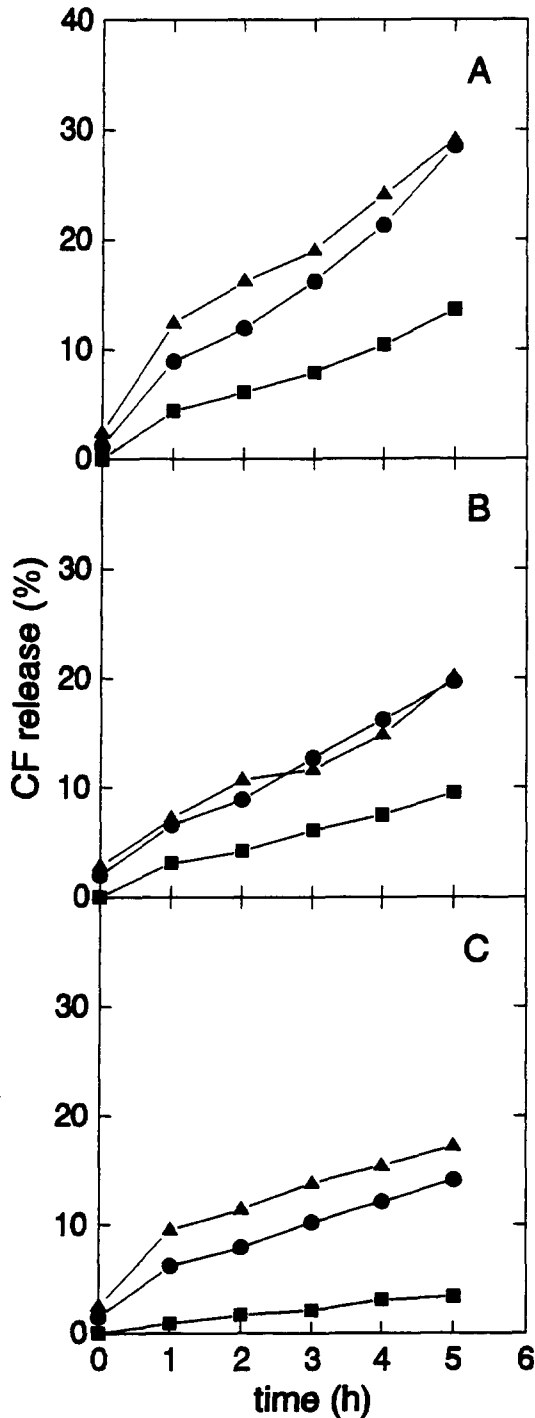


Fig. 5. Stability of liposomes at high temperatures. CF-loaded liposomes in 50 mM K-phosphate (pH 7.5) were incubated at 50, 65 and 80°C as indicated. The release of CF was measured during 5 h at 50°C (■ — ■), 65°C (● — ●) and 80°C (▲ — ▲). (A) *E. coli* lipids; (B) *B. stearothermophilus* lipids; (C) *S. acidocaldarius* lipids.

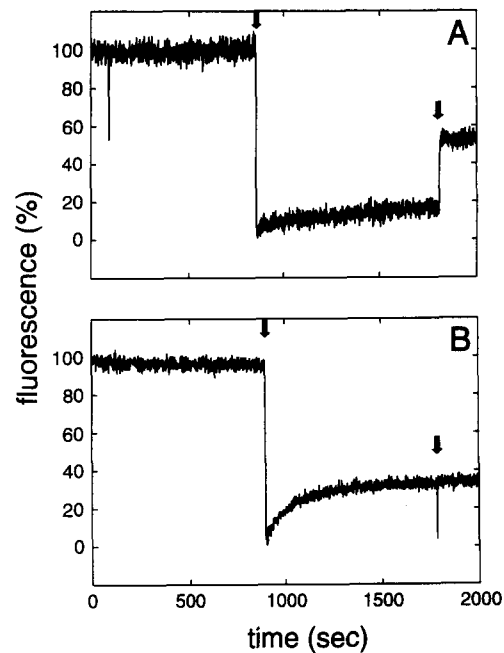


Fig. 6. Proton permeability in liposomes of *S. acidocaldarius* and *E. coli* lipids at 20°C. Liposomes were constructed as described in Materials and methods and diluted to a concentration of 1.5 mg lipid/ml in the external buffer. Subsequently valinomycin (1 nmol/mg lipid) was added. The fluorescence of pyranine, present at a concentration of 10  $\mu$ M, was monitored.  $H^+$  (100 nmol) and nigericin (50 nmol/mg *S. acidocaldarius* lipids or 1 nmol/mg *E. coli* lipids) were added as indicated by arrows 1 and 2. (A) *S. acidocaldarius* lipids; (B) *E. coli* lipids.

temperature, the liposomes prepared from *S. acidocaldarius* lipid were found to be the most stable, while *B. stearothermophilus* liposomes exhibited an intermediate stability.

### 3.3. Proton permeability of the liposomes

The proton permeability of the three different liposomal preparations was measured at increasing temperature. Liposomes prepared with a high buffer capacity on the inside were diluted in a solution with low buffer capacity. The external pH was monitored through the use of the fluorescent pH indicator pyranine. Liposomes were equilibrated in the suspending medium, and the external pH was lowered by a pulse of 100 nmol of protons (Fig. 6, first arrow) resulting in a rapid drop of the external pH. The external pH then gradually increased due to the influx of protons into the liposomes. Proton equilibration was obtained after the addition of the ionophore nigericin (Fig. 6, second arrow). These experiments were conducted in the presence of valinomycin and potassium ions to prevent the formation of a reversed membrane potential due to the electrogenic flux of protons into the liposomes. The proton-permeability of *S. acidocaldarius* liposomes (Fig.

6A) was found to be dramatically lower than that of the *E. coli* liposomes (Fig. 6B). The proton permeability was calculated by fitting the slow increase of the pyranine fluorescence after the proton-pulse to a single exponential. After each experiment, the pyranine fluorescence was calibrated through the addition of small aliquots of acid and base to the liposomes in the presence of nigericin. The  $H^+$ -permeability was measured over a wide temperature range. Little difference in proton permeability was observed between liposomes composed of lipids from *E. coli* and *B. stearothermophilus* (Fig. 7). However, at 40°C, the  $H^+$ -permeability of *S. acidocaldarius* liposomes was drastically lower than that of *E. coli* and *B. stearothermophilus* liposomes. Below 40°C, the  $H^+$ -permeability of the monolayer liposomes was virtually undetectable. At 20–30°C, liposomes of the mesophilic and thermophilic bacteria exhibit a comparable  $H^+$ -permeability as tetraether liposomes at 70–80°C. These results demonstrate that the monolayer liposomes of *S. acidocaldarius* lipid are equipped with an extremely low  $H^+$ -permeability.

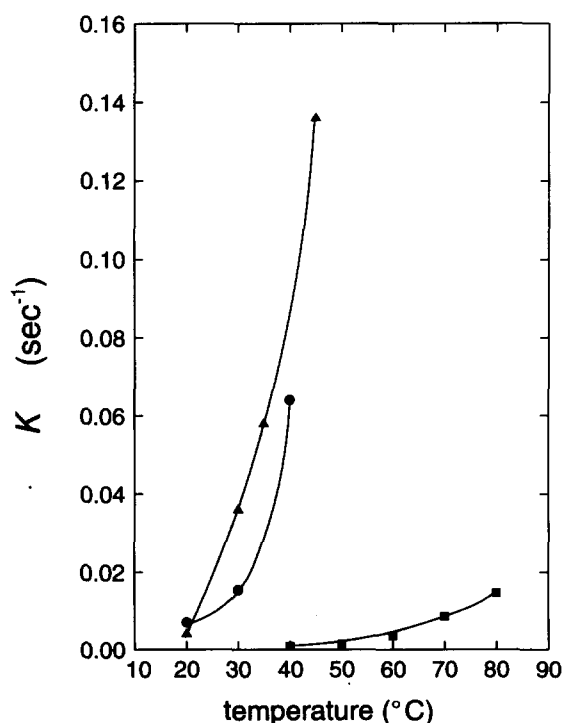


Fig. 7. Proton permeability in liposomes of *E. coli* lipids, *B. stearothermophilus* lipids and *S. acidocaldarius* lipids at increasing temperatures. Rate constants resulting from curve fitting of the fluorescence data as in Fig. 6 were plotted as a function of temperature. The standard error in the different experiments varied between 5 and 10%. *E. coli* lipids (●—●), *B. stearothermophilus* lipids (▲—▲) and *S. acidocaldarius* lipids (■—■).

#### 4. Discussion

In this paper we show that monolayer liposomes prepared from lipids extracted from the extreme thermoacidophilic archaeon *S. acidocaldarius* are very stable and have an unusually low proton permeability. The liposome-forming fraction contains mainly phospholipids and phosphoglycolipids. Conditions leading to the formation of vesicles of bipolar lipids of the related organism *Sulfolobus solfataricus* were recently investigated [9]. Liposomes composed of bipolar lipids could be obtained only if the amount of bisubstituted molecules (phosphoglycolipids) was increased. Bisubstituted lipid molecules, in which P-*myo*-inositol phosphate and one or two sugar residues are linked to opposite sites of the lipid, are thought to stabilize vesicle formation. The unsubstituted glycerol head group in monosubstituted molecules is poorly hydrophilic and might partition in the apolar core of the membrane [9]. This is in accordance with our observations where a relative enrichment of phosphoglycolipids leads to the formation of the stable liposomes.

The cryo electron micrograph of Fig. 3 clearly indicates that liposomes obtained by freeze-thaw extrusion are unilamellar. Freeze-fracture electron microscopy has already indicated that the tetraether lipids form a monolayer with both polar heads residing at opposite sites of the membrane [6]. This is in accordance with observations that synthetic tetraether amphiphiles primarily adopt a membrane-spanning configuration upon hydration [20]. The original multilamellar organization of these artificial liposomes was transformed into a unilamellar one upon aging, heating or sonication above the cmc of these lipids [20]. These data are consistent with the liposomal behavior of the naturally occurring archaeal lipids.

The properties of the monolayer liposomes have been compared with those of liposomes formed from phospholipids extracted from the mesophile *E. coli* and the thermophile *B. stearothermophilus*. The latter organism grows at 65°C, and its cytoplasmic membrane contains phospholipids with highly saturated acyl chains that give the membranes the proper fluidity at elevated temperatures [21]. From the ability to retain the fluorophore carboxyfluorescein it is evident that monolayer liposomes have a substantially higher stability at room temperature. Also at higher temperatures the stability is higher, but the differences with the bacterial phospholipid vesicles are less pronounced. Liposomes prepared from *S. acidocaldarius* lipids are essentially resistant to phospholipase A<sub>2</sub> activity (data not shown). This property is in accordance with the lack of ester bonds in tetraether lipids. As a result the monolayer liposomes will be more stable in biological fluids containing such enzymes (see also below).

Direct measurements of the proton permeability

demonstrate that the liposomes of *S. acidocaldarius* lipids are extremely well sealed for protons even at high temperatures. Studies on the proton permeability of small unilamellar vesicles composed of the synthetic 1,2-diphytanyl-*sn*-glycero-3-phosphocholine (DPhyPC) [22] indicate that the diphytanyl backbone is an important determinant in reducing the proton permeability of the membrane. At 20°C, the proton permeability coefficient of DPhyPC liposomes differs a factor 2 to 7 from that of conventional lipids [22]. It is difficult to directly compare the results as reported in Yamauchi et al. [22] with our data as these authors used an extreme pH shock, i.e., pH range 8 to 5, as compared to the subtle pH change used in this study (around pH 7.0). Moreover, the liposome diameter varies in both studies, making it necessary to determine the exposed lipid surface area in order to calculate true proton permeabilities. Our comparative studies indicate that liposomes formed from the natural tetraether lipid mixture decrease the proton permeability to a much greater extent. It will be of interest to study the contribution of the different structural aspects of the archaeal lipids to the relative impermeability of these membranes for protons.

The H<sup>+</sup>-permeability of liposomes of *E. coli* or *B. stearothermophilus* lipid did not differ significantly and both increased with increasing temperature. This observation is in line with the previous observation that thermophiles increase their proton permeability considerably at elevated temperatures [23]. The aerobic thermophiles have been found to compensate for the high proton permeability at their optimal growth temperature by increasing the proton pumping capacity in order to maintain a high protonmotive force [23]. Another adaptation to allow for chemiosmotic energy-transduction at higher growth temperatures is to use sodium ions instead of protons as the primary energy-coupling ion [24–28]. In the extreme thermoacidophile *S. acidocaldarius*, however, a completely different picture emerges. These cells grow in an acid environment, and their cytoplasmic membranes have to be tightly closed for protons in order to be able to maintain an intracellular pH of about 6.5. These cells use a classical chemiosmotic mechanism of energy coupling [29–32] based on a proton-translocating F<sub>0</sub>F<sub>1</sub>-type ATP-synthase and cytochrome-linked electron transfer chain. Our data suggest that one major adaptation of the cells to sustain the extreme environmental conditions is dictated by their unique membrane structure and composition. The low proton-permeability of these membranes protects the cells against acid damage and allows the cells to generate a high protonmotive force. It is likely that also other energy-transducing processes in the cytoplasmic membrane of *S. acidocaldarius*, such as secondary solute transport systems, use protons as coupling ions but this aspect has not yet been studied.

Additionally, cells gain their rigidity of the cell envelope by an S-layer. This S-layer covers the outer face of the cytoplasmic membrane and consists of oligomeric units of a single species glycoprotein that is anchored in the cytoplasmic membrane [33–36].

The high chemical and mechanical stability of the tetraether liposomes is a direct consequence of the chemical structure of these lipids and their monolayer organization. The lateral diffusion of the lipids in this monolayer is highly restricted [37]. In vivo, the sugar residues of the (phospho-)glycolipids are exposed at the outer face of the cytoplasmic membrane. A network of hydrogen-bonds between the sugar residues of individual lipid species limits the lateral diffusion of the lipids, thereby providing rigidity to the cytoplasmic membrane. Another aspect that may contribute to the stability is the introduction of up to four cyclopentane rings at high growth temperatures [5].

The *S. acidocaldarius* lipids open attractive experimental possibilities. The unusual low proton-permeability of the tetraether liposomes makes them very useful for the analysis of single (or limited) turn-over events of primary and secondary transport systems. Highly restricted proton backflow in such a system will facilitate the detection of translocated protons at the very early stages of a catalytic cycle. These liposomes now also allow us to reconstitute energy-transducing enzymes from *S. acidocaldarius* and to analyze their protonmotive force-generating properties at physiological temperatures (Gleißner, M., Elferink, M.G.L., Driessen, A.J.M., Konings, W.N. and Schäfer, G., in preparation). The high chemical and mechanical stability of tetraether lipid liposomes might be of advantage in their use as drug-carriers, either intra- or extravenous. Phospholipase A<sub>2</sub> has no effect on the integrity of these liposomes, which may be beneficial in drug-targeting via the skin in dermatological applications [38–40]. When phospholipid vesicles penetrate into the intact skin, they are unable to pass the horny layer due to early degradation by phospholipases [38]. The effectiveness of the tetraether lipid liposomes to pass this layer is currently being studied. Due to the high stability of the liposomes, a single treatment with drug-loaded liposomes may result in long-term protection as the drug is expected to be released only slowly. Another potential application of the liposomes is oral drug delivery. Since the tetraether lipids are adapted to low pH values, they may pass the stomach undamaged. These liposomes will only be of use as drug-carriers when at a later stage of circulation in the body, the permeability barrier can be disintegrated.

In conclusion, our data demonstrate that tetraether liposomes are more stable than conventional phospholipid liposomes prepared from natural sources. The extremely low H<sup>+</sup>-permeability of these liposomes makes them very useful in quantitative studies on H<sup>+</sup>-

translocating biological reactions and offers attractive possibilities for applications as drug-carrier.

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