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Monolayers of ether lipids from archaebacteria

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Abstract. The surface behavior of six different ether lipids from archaebacteria, based on condensation of glycerol or more complex polyols with two isoprenoid alcohols at 20 or 40 carbon atoms, was investigated in monolayers at the air-water interface.

The compounds with no complex polar group (GD, GDGT, GDNT) form monolayers showing a reversible collapse at surface pressure as low as 22 dynes/cm. This collapse pressure decreases with temperature in such a way that the film tension remains constant. In condensed films, these molecules do not assume a completely upright position.

Lipids with complex polar ends (HL, GLB, PLII) form films more stable to compression. Force-area characteristics and surface moment values of HL monolayers are similar to those of analogous ester lipids with fatty acid chains. Monolayers of the two bipolar lipids, GLB and PLII, at room temperature present a more condensed state, probably due to the lateral cohesion between long alkyl chains, but a lower collapse pressure.

For all bipolar lipids, the area expansion induced by temperature increase is larger than that of monopolar ones.

Key words: Archaebacteria, ether lipids, bipolar lipids, air-water interface monolayers

Introduction

Phylogenetic analysis of ribosomal RNA sequences (Woese and Fox 1977; Woese et al. 1978; Woese 1981; Tu et al. 1982), RNA polymerase (Zillig et al. 1982), tRNAs (Gupta and Woese 1980), 5SrRNA (Luehrsen et al. 1981), cell walls (De Rosa et al. 1974; Kandler and Konig 1978) and lipids (Kates and Kushwaha 1978; Langworthy 1978; Tornabene and Langworthy 1979; De Rosa et al. 1980a) support a division of bacteria that might have occurred early in the genealogical tree. According to this view, the living systems can be classified into three major phenotypes: eubacteria, archaebacteria and eukaryotes. The archaebacteria currently consist of methane-generating bacteria (methanogenes), a group of salt-tolerant bacteria (halophiles), and some extreme thermophiles. The lipids of archaebacteria are distinguished from those of the eubacteria and eukaryotes by the absence of fatty acid glycerol ester lipids. All of the polar lipids of archaebacteria are based on ether linkages, obtained by condensation of glycerol or more complex polyols with two isoprenoid alcohols at 20, 25 or 40 carbons atoms.

In Fig. 1 are reported the structures of some isoprenoid ethers (GD, GDGT, GDNT), basic structural elements of complex lipids of archaebacteria.

Extremely halophilic archaebacteria of genus *Halobacterium* and *Halococcus* possess lipids only based on the diether GD, while the tetraether lipids are the major lipid components of the membranes (90% ~ 95%) of the thermophilic archaebacteria of genus *Thermoplasma* and *Sulfolobus* (Langworthy et al. 1982; De Rosa et al. 1983). In particular, in *Sulfolobales*, the tetraethers with two and three cyclopentanes per biphytanyl chain are predominant (structures *b* 3; 4; 8; 12; 13; 17 in Fig. 1), the mean number of cycles depending on the growth temperature of the strain (De Rosa et al. 1980b).

Owing to the extreme conditions of habitat of the thermophilic archaebacteria, their lipids and membranes are supposed to have peculiar physical and structural properties, which have been examined with microcalorimetry on bulk phase (Gliozzi et al.

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Abbreviations: GD, Glycerol diether (2,3-di-O-phytanyl-*sn*-glycerol, GDGT, Glycerol-dialkyl-glycerol tetraether, GDNT, Glycerol-dialkyl-nonitol tetraether, GLB, Glycolipid B, PLII, Phospholipid II, HL, Total lipid extract from *Halobacterium halobium*

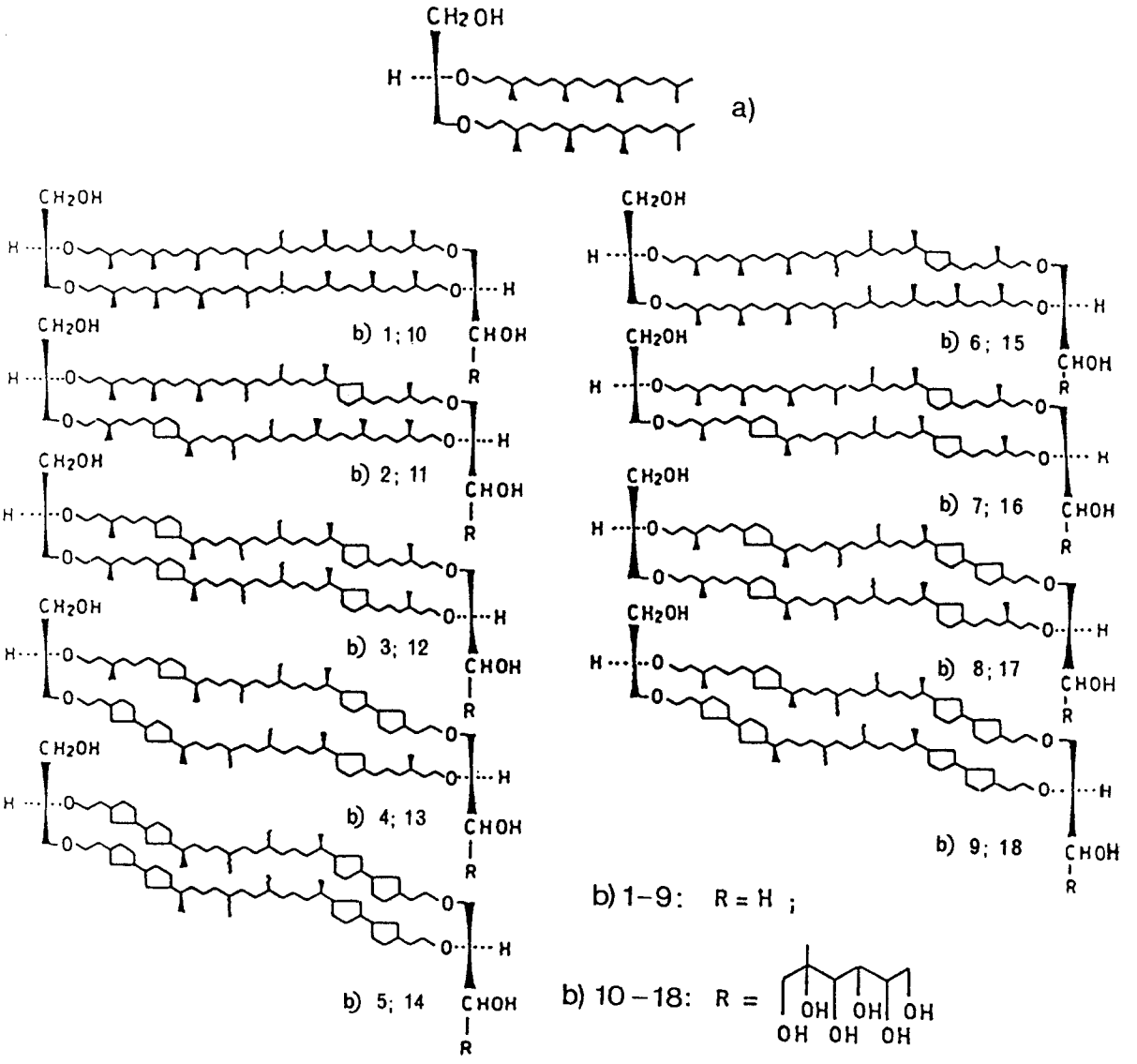

Fig. 1. Basic components of membrane lipids of archaebacteria, (*a*) Glycerol diether (GD), (*b 1–9*) Glycerol-dialkyl-glycerol tetraethers (GDGT). (*b 10–18*) Glycerol-dialkyl-nonitol tetraethers (GDNT)

1983 b; Blocher et al. 1984), electrical measurements on black membranes (Gliozzi et al. 1982, 1983 a), fluorescence spectroscopy on liposomes (Lelkes et al. 1983) and X-ray scattering techniques (Gulik et al. 1985). This work concerns the behavior of four bipolar lipids and two monopolar lipids from archaebacteria at the air-water interface.

Experimental

The four bipolar ether lipids, all obtained from *Sulfolobus solfataricus* are: glycerol-dialkyl-glycerol tetraethers (GDGT), glycerol-dialkyl-nonitol tetraethers (GDNT), Glycolipid B (GLB) and Phospholipid II (PLII). In all of these compounds, the lipophilic portions are C_{40} residues, with a mean of $2.3 \sim 2.5$ cyclopentanes per chain. In GDGT, the hydrophilic portions are two glycerol units (Fig. 1 structures *b* 1–9), while, in GDNT, one glycerol is replaced by a unique branched-chain nonitol (Fig. 1, structures *b* 10–18). The GLB is a 1-β-glucopyranosyl derivative of GDNT (Fig. 2 a). The PLII is a mixture of two glyco-phosphomyoinositol derivative of GDNT (Fig. 2 b) and of GDGT (Fig. 2 c). The GLB corresponds to 10.5% of the total lipid weight of *Sulfolobus solfataricus*, the two components of PLII are 42% (Fig. 2 b) and 6% (Fig. 2 c) respectively.

Monopolar ether lipid fraction (HL) extracted from *Halobacterium halobium* contains the diphytanylglycerol ether analogs of phosphatidylglycero-

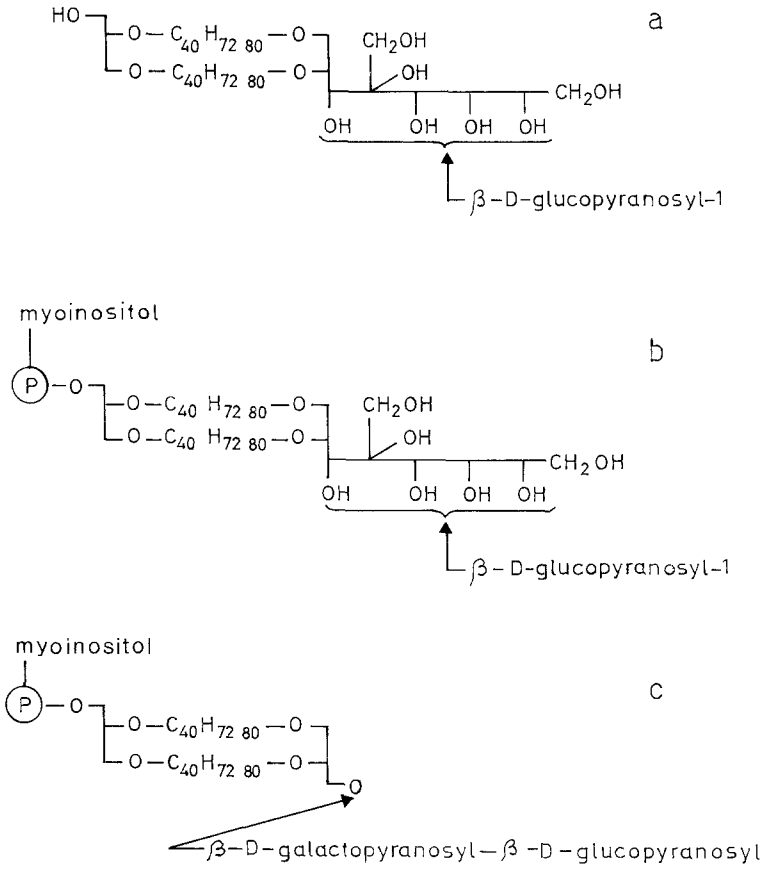

Fig. 2. Complex lipids of *Sulfolobus solfataricus*. **a** Glycolipid B. **b** and **c** Components of the Phospholipid II. **b** is 42% and **c** 6% of the total lipid extract

phosphate (PGP; 65%), phosphatidylglycerosulfate (PGS; 4%), phosphatidylglycerol (PG; 4%) and a SO-Gal-Man-Glc-di-O-phytanylglycerol (GLS; 25%) (Kates and Kushwaha 1978).

Sulfolobus solfataricus, strain MT-4, was grown at 87 °C, as described by De Rosa et al. 1975.

The glycolipids were extracted, separated and characterized according to De Rosa et al. (1980 a, b). GD, GDGT and GDNT have been obtained by hydrolyzing the total lipid extract with methanolic HCl for 6 h under reflux (De Rosa et al. 1983).

Dr. D. Oesterhelt of the Institut für Biochemie der Universität of München kindly provided the polar lipids of *Halobacterium halobium*.

Lipids were prepared in solutions of 2 mg/ml. GD, GDGT, GDNT, HL were dissolved in *n*-hexane or chloroform, GLB in chloroform and PLII in a mixture of chloroform, methanol and water (65 : 25 : 1). All the solvents were Analytical Reagent grade. An amount of these solutions (10 or 20 µl) was spread at room temperature (22–23 °C), by a Hamilton microsyringe or a micropipette, on a buffer solution (100 m*M* NaCl, 2 m*M* Hepes, pH = 7.4) prepared with bi- or tri-distilled water. This solution was degassed for measurements as a function of temperature. The water surface had been previously cleaned several times. Five to ten minutes after the spreading force-area curves were automatically obtained on a X-Y recorder by a Monofilmmeter (type RCM2-T, Mayer-Feintechnik, Göttingen) constructed according to Fromherz (1975). Usually in the compression, the film area changed from 340 to 20 cm^2. The trough of the Monofilmmeter was thermostatted and situated in a cupboard to eliminate draughts and dust particles. For measurements at high temperature, a compartment of thermal insulating material was built around the trough. To avoid steam condensation, the air temperature in the compartment was maintained a few degrees higher than that of the aqueous subphase by an electrical resistor. Air and subphase temperatures were recorded, and their mean value was assumed as the temperature of the surface.

The surface potential was measured by an electrometer connected to two air-ionizing electrodes (Am 241) suspended at about 5 mm from the monolayer and free salt solution surface. For this type of measurement, films were spread on a glass trough of 300 cm^2 area, the compression was carried out discontinuously by a teflon bar while the surface pressure was obtained according to the well-known Wilhelmy method, measuring the force on a thin microscope glass plate by an electrobalance (Mettler). Potential was read within a 3 minute interval

during which it varied by 5% around the mean values.

Results and discussion

All of the ether lipids examined change the surface tension of a buffer solution when spread by a volatile solvent. The trends of the surface pressure isotherms, the values of the surface potential and the general behavior, suggest the formation of insoluble monomolecular films. On the basis $\pi - a$ characteristics, two different types of film may be identified: those formed by the compounds having glycerol or nonitol as polar groups (GD, GDGT, GDNT) and those with a more complex polar group at least (HL, GLB, PLII).

The films of the first type are not stable. The aging and subsequent compressions and decompressions cause the increase of the film area (Fig. 3). This effect is independent of the spreading solvent (*n*-hexane or chloroform) and it is also present when the experiments were performed in an Argon atmosphere. When the lipid solutions are sonicated for a few minutes, more expanded films are obtained; however, the expansion effect is still present. It must be pointed out that GDGT and GDNT films expand more than GD film. Furthermore, the curve *a'* of Fig. 3 refers to an almost steady state, while the GDGT and GDNT isotherms continue to expand even after 2 h.

Every force-area characteristic of Fig. 3 describes a complete compression-decompression cycle and shows hysteresis, whose amplitude is wider for more expanded films and depends on the minimum area reached in compression.

The bending of the curves at 21 dynes/cm has to be attributed to a reversible transition to a bulk phase, because the following plateau extends to very small areas incompatible with a monomolecular structure.

Notwithstanding the instability of the films, the first compression curves show good reproducibility, the maximum deviation from the mean values of the area per molecule at $\pi = 10$ dynes/cm are 2% for GD (5 measurements), 5% for GDGT (3 measurements) and 8% for GDNT (5 measurements).

The minimum of compressibility of the just spread films of GD, GDGT and GDNT, referred to curves *a*, *b*, *c*, of Fig. 3, are 1.2×10^{-2} cm/dyne at $\pi = 18$ dynes/cm, 4.2×10^{-3} and 6.8×10^{-3} both at $\pi = 14$ dynes/cm. These values change with time and/or repeated compressions and from curves *a'*, *b'*, *c'* one obtains 1.5×10^{-2}, 1.0×10^{-2} and 1.0×10^{-2}.

We have little supporting evidence to explain the instability of these films. Chemical stability of these compounds, ineffectiveness of inert atmosphere on the phenomenon and its absence in monolayers of analogous ether lipids makes chemical degradation improbable. The effect of sonication suggests that the compounds are not perfectly solubilized by the solvents used (chloroform and *n*-hexane) and the molecular aggregates present in the spreading solution take time and energy, furnished by repeated compressions, to be transformed into a monolayer. On the other hand the polar groups can interact. Intermolecular hydrogen bonding is possible. If the resulting gathering of the polar heads would hinder the vertical alignment of the molecules to the plane of the surface, it could explain the increase in average area with time.

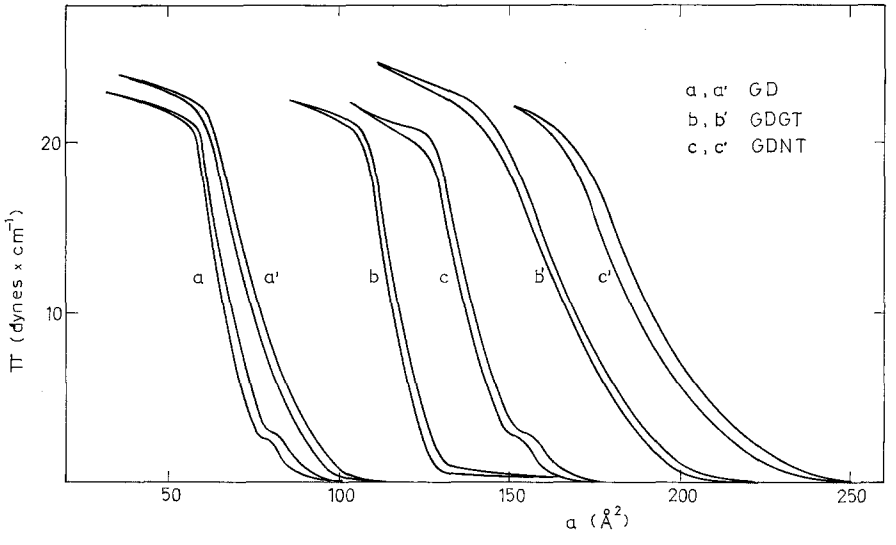

Fig. 3. Surface pressure-area per molecule isotherms of GD, GDGT and GDNT monolayers on NaCl 100 m*M*, Hepes 2 m*M*, pH = 7.4. Room temperature 22–23 °C. (*a*) GD, first compression-decompression. (*a'*) GD, 7th compression-decompression, 1 h after the spreading. (*b*) GDGT, first compression-decompression. (*b'*) GDGT, 7th compression-decompression, 2 h after the spreading, the hexane solution was sonicated for a few minutes before the spreading. (*c*) GDNT, first compression-decompression. (*c'*) GDNT, 7th compression-decompression, 1 h after the spreading, the hexane solution was sonicated for a few minutes before the spreading. All the isotherms were carried out at a rate of 1.1 dynes/min

Time dependent compression isotherms and surface potential values at different pH and at different salt concentration of the substrate should provide evidence of this possibility. These measurements are in progress.

Adam and Jessop (1926) examined monomolecular films of long chain molecules with a polar group at each end and showed that di-ethyl esters with 16, 20 and 32 carbon atoms at low pressure lie flat on the water surface forming gaseous films, while at higher pressures form condensed films with molecules standing upright, with only one polar end adhering to the water.

Considering molecular sizes, such a position in the condensed films seems unreal for GDGT and GDNT molecules. GD molecules also do not assume a complete vertical alignment to the plane of the surface in a rigid crystalline state.

According to space-filling models, the cross-section area of the alkyl chains in a trans configuration (26 Å^2) exceeds the area of the glycerol and also of the nonitol, if the latter is also in a trans configuration parallel to that of the hydrophobic chains. Gaines (1966) indicates a requirement of 22 to 29 Å^2/molecule, at the highest compressions of films of compounds having one branched chain and furnished a limiting area of 24 Å^2 of Phytol (3,7,11,15-tetramethyl hexadecene-2-ol-1) and Phytanic acid (3,7,11,15-tetramethylexadecanoicacid).

Therefore the vertical double chained molecule of GD requires 48 Å^2 for a trans configuration in a rigid crystalline state while an area of ~ 70 Å^2 is expected for the liquid crystalline state. Figure 3a shows an area per molecule of 93 Å^2 at the pressure of 0.5 dynes/cm, where a sharp bend indicates the curve for the condensed film is produced and an area of 69 Å^2, just before the collapse (Fig. 3a). This last value indicates that the monolayer collapses directly from the liquid state.

Cycles increase the area of a chain in bipolar compounds up to 30 Å^2 (Gulik et al. 1985) and then the upright position of GDGT and GDNT should require 60 Å^2, much smaller than the areas reported in Fig. 3b, c.

For bipolar compounds we must consider a packing in which both polar ends of the molecules are in the water, the chains forming an arch between the polar groups (*n*-shaped configuration). Such packing should occupy an area of at least 120 Å^2 per molecule, and the arch might easily require considerably more, owing to the impossibility of bending a branched hydrocarbon chain very sharply. If we assume no molecular aggregate in the monolayers, the area values for the condensed films of Fig. 3b, and c indicate that a proportion of GDGT and GDNT molecules should be in the *n*-shaped configuration and the trends of the curves *b'* and *c'* suggest that a greater number of molecules are adhering by both ends to the water surface and/or are assuming a less sharply bended configuration. The larger areas occurring in the GDNT force-area characteristics in respect of GDGT ones support the hypothesis of a configuration with the nonitol not dipping in the subphase, because only in this case can it contribute appreciably to the occupied area.

Large fluctuations of the surface potential are usually observed in non-homogeneous films (Gaines 1966). Therefore the stability of the surface potential values of all the examined films (see Experimental) seems to prove their homogeneity at least on a macroscopic scale.

The surface potential plotted versus surface pressure of Fig. 4 is referred to the first compression (curves *a*, *b*, *c* of Fig. 3) and the corresponding surface moment, the component of the molecular dipole moment perpendicular to the water surface is calculated according to

$$p = \varepsilon_0 V \times a ,$$

where ε_0 is the dielectric constant of the vacuum, V is the measured surface potential and a, the area per molecule (Shah 1972). The overall dipole moment results from the contribution of the dipole moments μ_1 from the orientated water molecules, μ_2 from the polar head groups and μ_3 from the hydro-

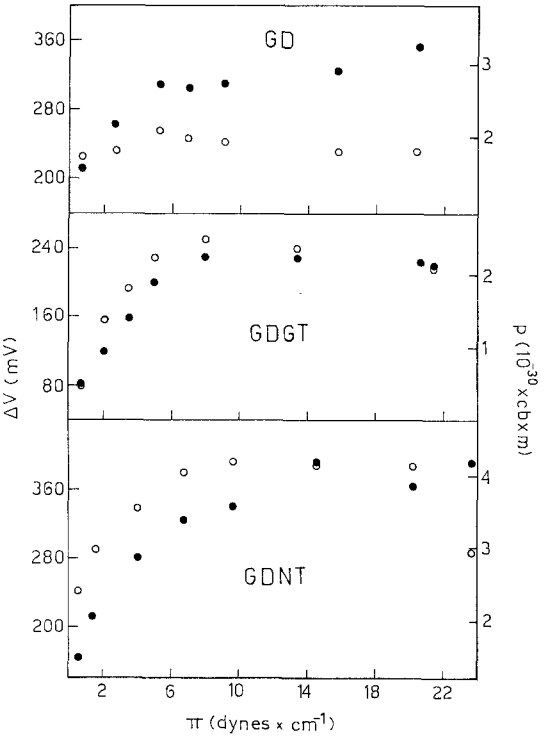

Fig. 4. Surface potential (●) and surface moment (○) of GD, GDGT and GDNT versus surface pressure. See Fig. 3a, b and c for corresponding areas per molecule

carbon chains (Davies and Rideal 1963). These contributions are not readily separable, but indications on the molecular shape can be deduced from surface moment values and their behavior as a function of surface pressure and area per molecule.

Assuming that GD molecules in condensed monolayers are roughly upright, for a compact *n*-shaped configuration of GDGT molecules, as the area values of Fig. 3b suggest the GDGT surface moment must be expected to be double that of the GD one. Because the maximum experimental values (2.5×10^{-30} cb × m for GDGT and 2.2×10^{-30} cb × m for GD) do not fit with this idea, a more reasonable hypothesis is to suppose GDGT molecules in a random packing, a proportion standing upright and a proportion in *n*-shaped configuration with different degree of bending. The very high dipole moment of GDNT (4.3×10^{-30} cb × m) could be due to the great capability of the nonitol to bind water molecules (Gliozzi et al. unpublished results) increasing the μ_1 contribution.

The ether lipids with more polar groups form stable monolayers which present irreversible collapse at higher surface pressure (Fig. 5).

The trend of the force-area characteristic (Fig. 5a), surface potential and dipole moment (Fig. 6a) of the total polar lipid extract of *Halobacterium halobium* is very similar to the ester polar lipids (Gaines 1966; Maggio et al. 1978), more expanded at low pressure owing to hydrophilic hydrocarbon chains and limiting area per molecule, just before collapse, not much larger than the cross sectional area of two alkyl chains. GLB and PLII form more condensed films, possibly due to the double length of the hydrophobic chains.

The area values indicate that most of the bipolar molecules stand upright with one polar group in the water and the other one away from the surface. If one assumes, as for ester glyco-lipids, that limiting areas depend on hydrophobic chain dimensions (Maggio et al. 1978), the values of 75 Å^2 for GLB and 85 Å^2 for PLII, sensibly larger than the cross sectional area for two cyclized alkyl chains, do not exclude the presence of *n*-shaped molecules.

The minimum compressibility values are: HL, 1.4×10^{-2} cm/dyne at $\pi = 38$ dynes/cm, GLB, 4.8×10^{-3} cm/dyne at $\pi = 22$ dynes/cm, PLII, 5.7×10^{-3} cm/dyne at 21 dynes/cm. All these values are smaller than those found by Maggio et al. (1978) for gangliosides and glycosphingolipids, and the values for GLB and PLII are comparable to those of monolayers of various lecithins reported by Van Deenen et al. (1962).

Davies (1948) observed lower surface moments for bipolar compounds considered to be oriented vertically, with one polar group in the surface, in

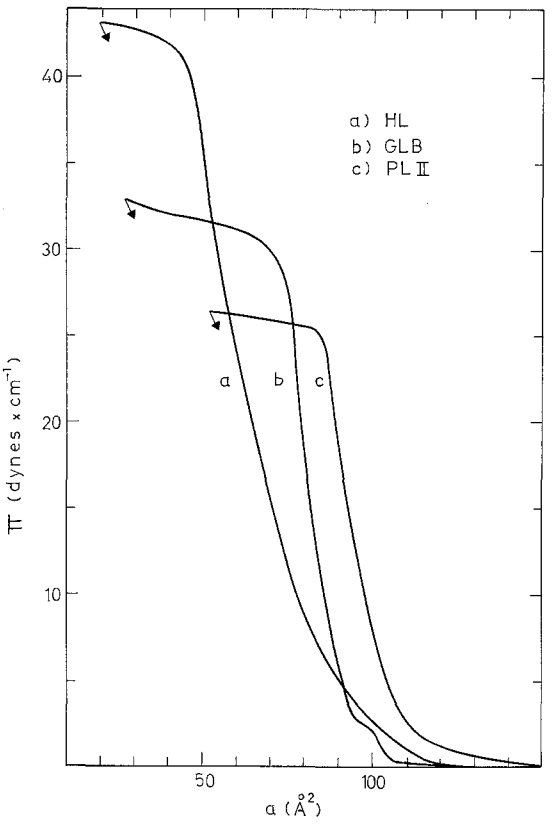

Fig. 5. Surface pressure-area per molecule isotherms. (*a*) Total lipid extract from *Halobacterium halobium*, (*b*) Glycolipid B, (*c*) Phospholipid II. *Arrows* indicate irreversibility of the collapse. Same subphase and temperature as Fig. 3

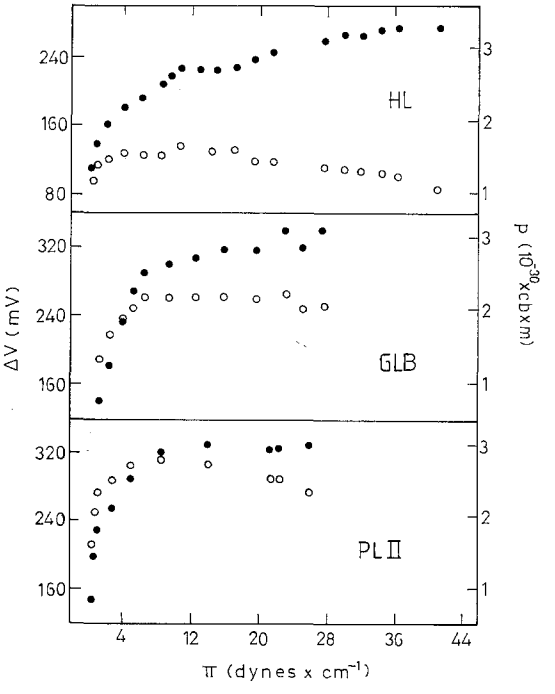

Fig. 6. Surface potential (●) and surface moment (○) of HL, GLB and PLII versus surface pressure. Corresponding areas per molecule in Fig. 5

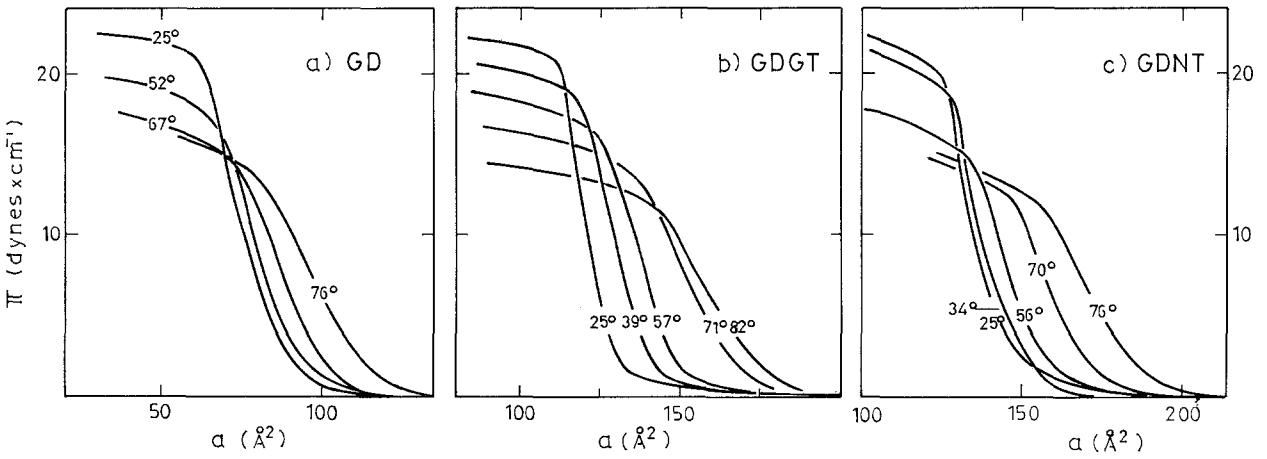

Fig. 7. Surface pressure-area per molecule isotherms of GD, GDGT and GDNT at different temperatures. Same subphase as Fig. 3

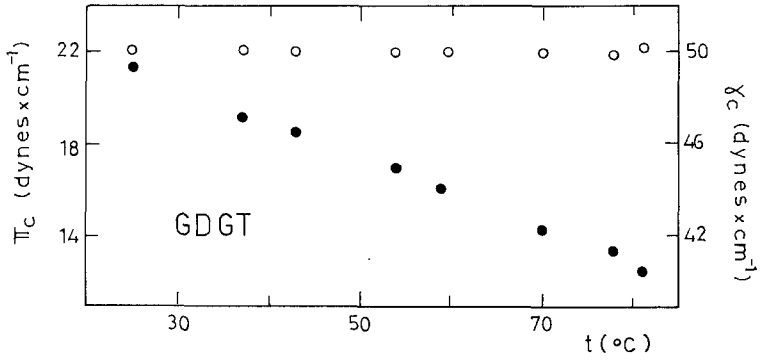

Fig. 8. Collapse pressure (●) and tension (○) versus temperature for GDGT films. The collapse pressure is taken at the intersection of two straight lines fitting the plateau and the higher part of the curve. GD and GDNT films present very similar values

comparison to those considered lying flat and a sensible decrease of the surface moment when the monolayer started to be condensed. By contrast, no change in monotonic increase of the surface potential with the surface pressure was observed for all the bipolar lipids examined (Fig. 4 and 6).

Temperature dependence

The different nature of films formed by the two groups of ether lipids is shown by force-area curves at different temperatures. Figures 7 and 8 show that the collapse pressures of less polar compounds decrease with temperature.

In Fig. 8 the pressure collapse of GDGT versus temperature is reported; the trend of the analogous curves for GD and GDNT is quite similar. One obtains the same curves with negligible hysteresis by decreasing temperature at a rate of 1 degree/min (about the same as the rate of heating). The corresponding values of the film surface tension at the collapse calculated according to

$$\gamma_f(t) = \gamma_w(t) - \pi(t),$$

where γ_f is the surface tension of the film, γ_w the surfaced tension of the free subphase, π the measured collapse surface pressure and t the temperature, are constant. They are 50.4 ± 0.6 dynes/cm for GD, 50.0 ± 0.4 dynes/cm for GDGT and 50.5 ± 0.9 dynes/cm for GDNT, the errors are standard deviations on ~ 40 observations.

The collapse pressures of GLB and PLII do not change very much with temperature. Collapse pressures at high temperature have about the same values as at room temperature (GLB, 27.7 dynes/cm at 80 °C, 30 dynes/cm at 22 °C, PLII 25 dynes/cm at 70 °C, 25 dynes/cm at 22 °C).

In Figs. 7 and 9, the greater expansion rate of molecular area of bipolar lipids in comparison with monopolar ones can also be noticed. It is probably due to the thermal agitation tending to disrupt the lateral cohesion between the upright molecules, and favours either a less definite or a horizontal orientation. The instability of GDGT and GDNT films makes the increase of the area per molecule at a certain pressure depend very much on the heating rate. The area reached at the maximum temperature is maintained if the temperature is very slowly de-

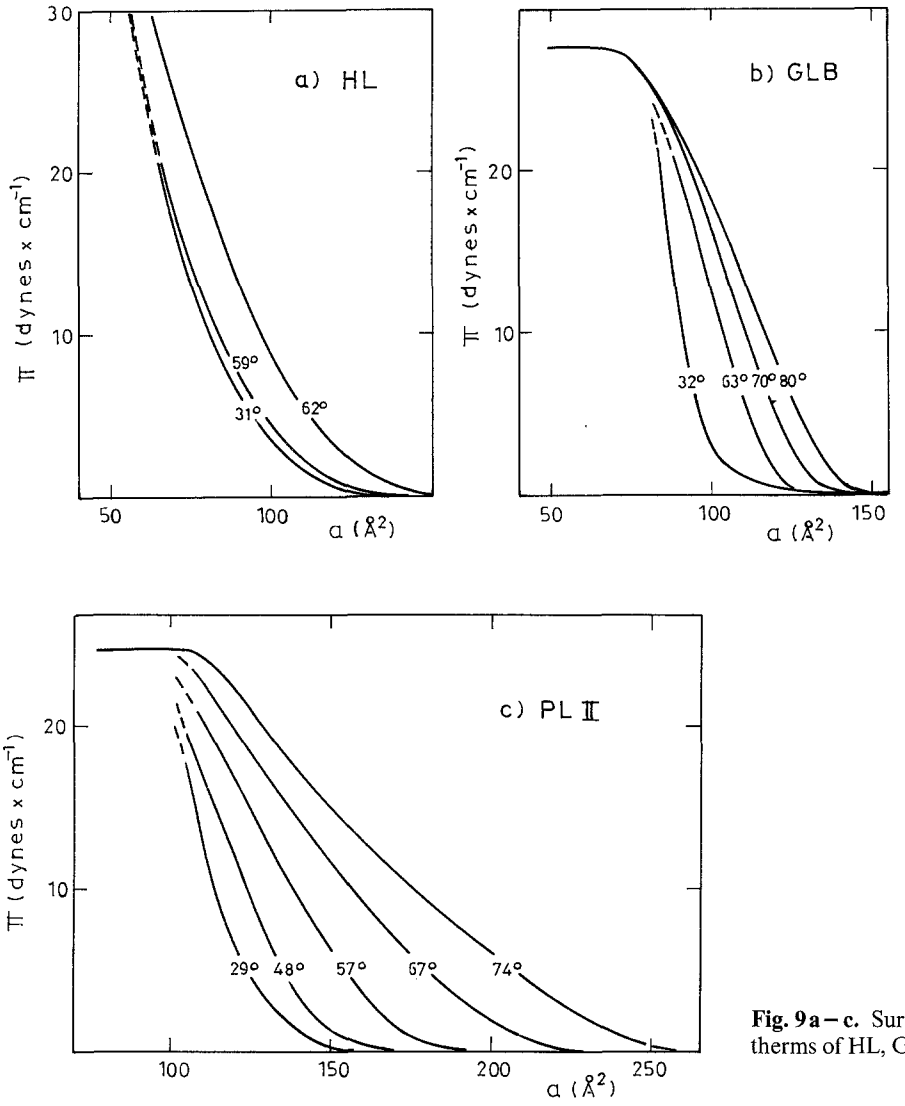

Fig. 9a–c. Surface pressure-area per molecule isotherms of HL, GLB and PLII at different temperatures

creased to room temperature. The HL monolayer is not significantly influenced by temperature in the range 20–50 °C like monolayers of egg-lecithin with unsaturated fatty acid chains, suggesting the same role of unsaturation and branching (Shah 1972).

GD, GDNT, GLB isotherms at room temperature reveal a phase transition at 3 dynes/cm. It vanishes with repeated compressions and for GD and GDNT does not occur above 25 °C and for GLB above 32 °C (Fig. 7). It was not further examined in this work.

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