BRIEF COMMUNICATION

ARTIFICIAL BLACK MEMBRANES FROM BIPOLAR LIPIDS OF THERMOPHILIC ARCHAEBACTERIA

Alessandra Gliozzi
Istituto di Cibernetica e Biofisica, Camogli (Ge)
Ranieri Rolandi
Istituto di Scienze Fisiche dell'Università di Genova, Italy
Mario De Rosa and Agata Gambacorta
Istituto per la Chimica di Molecole di Interesse Biologico, Arco Felice (Na), Italy

ABSTRACT The membrane of thermophilic archaebacteria is characterized by the presence of unusual isoprenoid bipolar lipids. The molecular organization of these lipids is still a matter of study. Important information could come from forming artificial black membranes. Black films can be formed from *n*-alkane or squalene dispersions of bipolar lipids extracted from the membrane of *Caldariella acidophila*. Membrane formation occurred only above a critical temperature (~70°C) corresponding to the physiological one. At lower temperatures, special solvent systems (*n*-alkanes or squalene, butanol and *n*-alkanes or squalene, butanol chloroform) were required. To characterize the physical parameters of these membranes, conductance and capacitance measurements were performed. Conductance was in the range of 10^{-8}–10^{-7} $\Omega^{-1}\text{cm}^{-2}$, where specific capacitance at $T = 72°\text{C}$ was $C_s = 0.685 \pm 0.004$ $\mu\text{F/cm}^2$ and $C_s = 0.658 \pm 0.08$ $\mu\text{F/cm}^2$, corresponding to a dielectric thickness of 27 and 29 Å for squalene and dodecane dispersions, respectively. Capacitance was shown to vary as the square of membrane potential, as usual in lipid bilayers. Values of the proportionality constant α have been compared to those of solvent-containing and solvent-free bilayers. The behavior of capacitance as a function of temperature is also shown by lowering temperature; the occurrence of complex structural changes was indicated. All the experimental data suggest that the presence of solvent is very low. Two possible molecular configurations of the films are discussed.

Lipid molecules were extracted from the membrane of the thermophilic bacteria *Caldariella acidophila* (1). The membrane of this microorganism, whose physiological tolerance is in the range of 62° to 90°C, is built up from lipids of varying complexity (2, 3) which, on hydrolysis, afford two main types of components. These are macrocyclic tetraethers where the two lipophilic portions are C_{40} residues and the skeletons are 16,16′-biphytanylglycerol with different degrees of cyclization (1, 2). In one tetraether, the hydrophylic portions are two glycerol units and in the other the hydrophylic portions are glycerol and a unique branched-chain nonitol (Fig. 1). We were able to obtain black films only with the latter.

The black membranes were prepared by a conventional technique (4), in which an air bubble was used to paint a submerged solution of a natural mixture of glycerol-dialkyl-nonitol tetraethers (GDNT) (25 mg/ml) in organic solvents (squalene or *n*-alkanes) over a circular hole (0.6–1.2 mm in diameter) in a teflon partition separating two aqueous solutions (0.1 M NaCl, unbuffered).

The kinetics of formation were controlled by temperature and solvents. At $T \simeq 75°\text{C}$ the formation of a black membrane required only a few seconds. Once formed the membrane lasted several hours and the temperature could be changed over a wide range (80–10°C) without membrane breakage. At lower temperatures, the formation of a membrane required specific solvent systems (squalene or *n*-alkanes, butanol 80:20 by volume [$\sim T \simeq 60°\text{C}$] and squalene or *n*-alkanes, chloroform-butanol 57:29:14 by volume [at $T \simeq 40°\text{C}$]). In such a case the formation could take several hours. The critical roles of the solvents and the temperature on the formation kinetics may be ascribed to the partial freezing of the C_{40} aliphatic chains.

The specific membrane conductance in 0.1-M NaCl was in the range of 10^{-8} to $10^{-7}\Omega^{-1}\text{cm}^{-2}$, as expected for a good insulator. However, at $T > 70°\text{C}$ this value was not stable, especially when high potentials (≥100 mV) were applied, and conductance increased. In contrast, stable values were obtained by lowering the temperature to $T \leq 60°\text{C}$.

The role of temperature in determining the physical characteristics of the membranes was investigated by performing precise measurements of specific membrane capacitance, C_s in the range of 80° to 10°C. Fig. 2 shows a typical behavior of a squalene-GDNT membrane. Such

Biophys. J. © Biophysical Society · 0006–3495/82/02/563/04 $1.00
Volume 37 February 1982 563–566

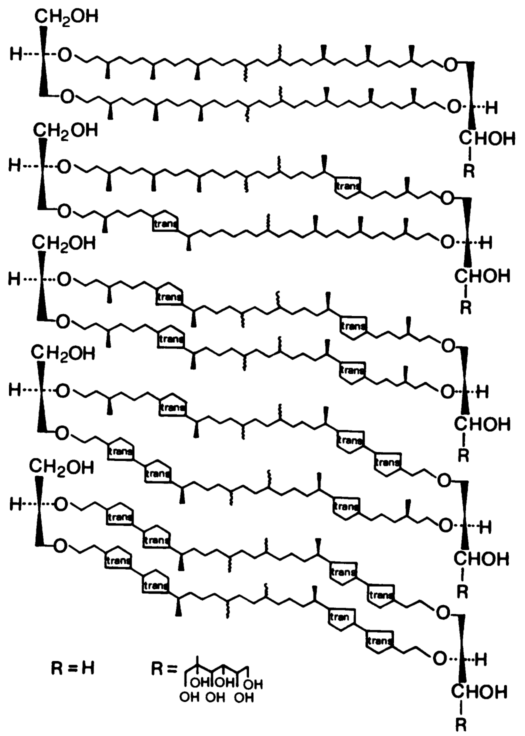
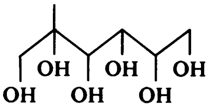


FIGURE 1 Glycerol-dialkyl-glycerol tetraethers (GDGT) (R = H) and glycerol-dialkyl-nonitol tetraethers (GDNT) R =  as basic components of membranes lipids in *Caldariella acidophila*.

behavior seems to indicate that several structural changes occur on decreasing temperature, the first one likely around $T \simeq 70°C$.

Capacitance measurements were also performed by dispersing the lipids in two different solvents: dodecane and squalene. These measurements compared the behavior of our films with that of the usual artificial bilayers (BLM) in two different solvent systems. In fact, it has been shown that the dielectric thickness of BLM depends on the type (5–8) and concentration (9) of solvent in which the lipid is dispersed. This feature is ascribed to the different amounts of solvent present in the mid-plane of the bilayer, which decrease on increasing the length of the alkyl chains. Moreover, very long *n*-alkane molecules (>21 carbons) and squalene were shown to be insoluble in glycerol monooleate (GMO) bilayers (10, 11). Membranes formed by these lipid dispersions should thus be considered virtually solvent free.

Results from capacitance measurements for bipolar lipids dispersed in squalene and in dodecane at $T = 72°C$ gave $C_s = 0.685 \pm 0.004$ and $= 0.658 \pm 0.008\ \mu F/cm^2$, for a dielectric thickness, d, of 27 and 29 Å, respectively. If the same conclusions drawn for the usual BLM can be extended also to the present system, the small difference in capacitance (and in dielectric thickness) between membranes formed from squalene and dodecane lipid dispersions seems to indicate that the solvent content is very low, even in the case of alkyl chains shorter than 21 carbons.

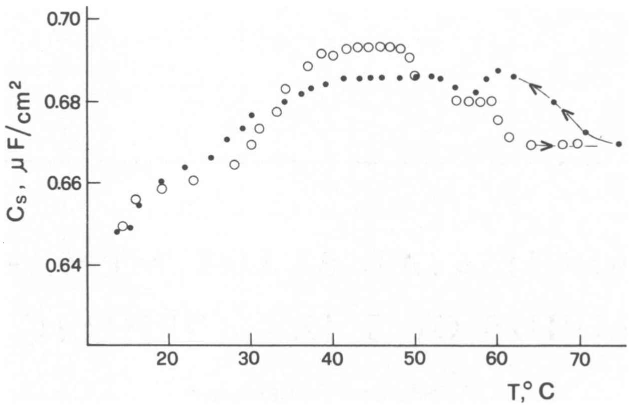


FIGURE 2 Specific membrane capacitance, C_s, is plotted as a function of temperature for a GDNT-squalene membrane. No addition of chloroform or butanol was present in these films. Ionic solutions were unbuffered 0.1 M NaCl. The chamber used for membrane formation was machined from a teflon block. It had optically flat windows in either end and was held in a copper thermostat. Temperature was monitored with a thermistor. Membrane capacitances were measured at 500 Hz [V_{AC}(max) = ± 50 mV] with a precision (0.1%) a.c. impedance bridge. Extrapolation to zero potential introduces negligible corrections in the values reported above. Photographic determinations of the membrane area were made using transmitted light. The error of the area was about 0.3%. Before each series of measurements, the membrane was allowed to "age" for 10–15 min under the action of a 50-mV continuous potential. Under these conditions time-invariant values of C_s were obtained. Temperature was lowered at a rate of ~1°C/min. (●) Pattern of decreasing temperature, (○) pattern of increasing temperature.

Table I compares results from GDNT membranes to the corresponding values of a GMO bilayer. Notice that we have compared the capacitance when both structures are in the liquidlike state, which occurs for GDNT and GMO membranes above 70° and 17°C, respectively (12, 13). Also reported in Table I is the value of α, a proportionality constant relating the capacitance at zero potential $C(0)$ to the capacitance at a potential $V, C(V)$, according to the relationship

$$C(V) = C(0)(1 + \alpha V^2). \qquad (1)$$

Eq. 1 was shown to hold both in solvent-containing (6, 14) and in solvent-free membranes (15) (made with lipids dispersed in squalene [11] or by hydrophobic apposition of two monolayers [16]). Results of Fig. 3 indicate that also in the present system a linear dependency of capacitance on the square of voltage occurs. However, as shown in Table I, the dependence on the employed solvent is much less dramatic than in the usual artificial bilayers.

As expected, the temperature seems to be a very important factor in determining the value of α. However, we could only obtain an upper estimate of the α value at lower temperatures. For instance for a GDNT membrane in

TABLE I

	Bipolar lipid membranes			Monoolein lipid membranes		
	C_s	d	α	C_s	d	α
	μF/cm²	*Å*	V^{-2}	*F/cm²*	*Å*	V^{-2}
Lipid dispersed in dodecane	0.654 ± 0.008	29	0.40 ± 0.05	0.400 ± 0.007	49	8.5 ± 0.6
Lipid dispersed in squalene	0.684 ± 0.004	27	0.16 ± 0.06	0.7771 ± 0.0048*	25	0.03*

Comparison of the specific capacitance, C_s, the dielectric thickness, d, and the proportionality constant of the capacitance-voltage relationship, α, of bipolar lipid membranes from glycerol-dialkyl-nonitol tetraether (GDNT) of *Caldariella acidophila* and bilayer membranes from glycerol monooleate (GMO) (Sigma Chemical Co., St. Louis, Mo.) dispersed in two different solvents, dodecane (BDH Chemicals Ltd., Poole, Eng.) and squalene (Sigma Chemical Co.). The values chosen to ensure the fluidity of the aliphatic chains were T = 25°C for BLM and T = 72°C for bipolar lipid membranes. The dielectric thickness of BLM was calculated using (8) $\epsilon_r = 2.2$, whereas that of bipolar lipid membranes was determined assuming $\epsilon_r = 2.1$, to account for the temperature dependence.

The capacitance-voltage relationship was determined superimposing a continuous potential V_{DC} to the 500 = Hz signal. After application of high potentials (≥100 mV) a slightly higher value of zero-voltage capacitance was often observed. To correct for this effect, zero-voltage capacitance was measured before and after any voltage application. Values of α were deduced (6, 14) by the best fitting of Eq. 1, where the applied potential V is given by $V = [V_{DC}^2 + 1/2\ V_{AC}^2\ (\max)]^{1/2}$. Other conditions are as those indicated in Fig. 2.

*Values from reference 11.

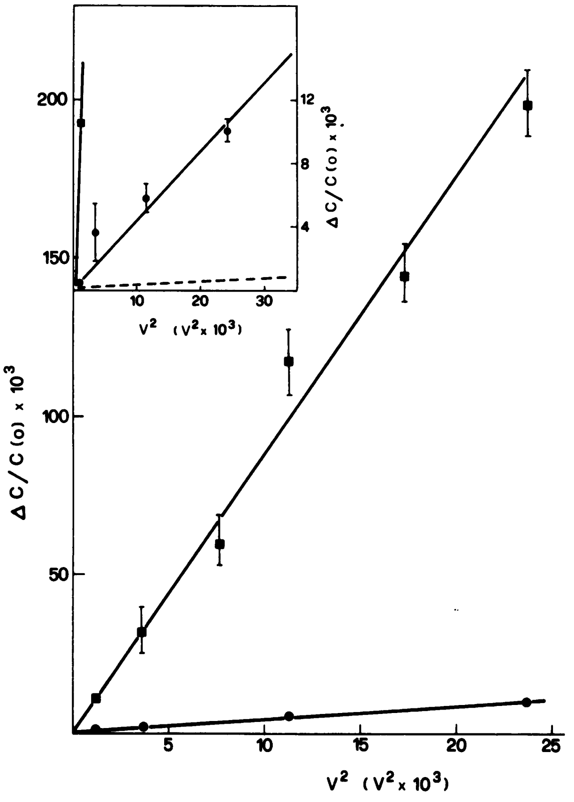


FIGURE 3 The relative capacitance variation ΔC/C(O) is reported as a function of the square of membrane potential $V = [V_{DC}^2 + \frac{1}{2} V_{AC}^2(\max)]^{1/2}$, for a 25-mg/ml dispersion of dodecane-monoolein membrane (■) and dodecane-glycerol-dialkyl-nonitol tetraether (●). The experimental conditions are as those indicated in Fig. 2. The straight lines are the best fitting of Eq. 1, with regression coefficient r = 0.98 and r = 0.99 for GDNT and GMO membranes, respectively. The bars are the standard errors on the points. The *inset* is a replotting of the data in a different scale (indicated on the right). The dotted line is the value $\alpha = 3.10^{-2} V^{-2}$, corresponding to squalene-monoolein membranes (11).

squalene at T = 42°C we got $\alpha \leq 5\ 10^{-2}V^{-2}$, a value in a range similar to that found for solvent-free membranes (11, 15). Any interpretation of the results reported above is linked to the fundamental question of how lipids are arranged in the membrane. Two possible models can be considered: a monolayer organization of the bipolar lipids or two apposed monolayers in which both polar heads, being anchored to the aqueous interfaces, induce a folding of the isoprenoidic chains (we may call this the U-shaped configuration). The experimental data reported above simply suggest that the presence of the solvent is very low, leading to squalene and dodecane membranes being roughly equivalent. Thus the lack of a mid-plane region where the solvent might be located in the case of a monolayer structure, or the inherent entropic restrictions of the alkyl chains, in the case of an U-shaped configuration, might both lead to such a behavior. Experiments aimed to distinguish between these two possible configurations are in progress.

We thank S. Ciani for a critical reading of a first draft of the paper. The technical help of E. Gaggero is also greatly acknowledged.

Received for publication 15 June 1981.

REFERENCES

1. De Rosa, M., S. De Rosa, A. Gambacorta, and J. D. Bu'Lock. 1980. Structure of calditol, a new branched-chain nonitol and of the derived tetraether lipids in thermoacidophile archaebacteria of the *Caldariella* group. *Phytochemistry.* 19:249–254.
2. De Rosa, M., S. De Rosa, A. Gambacorta, L. Minale, and J. D. Bu'Lock. 1977. Chemical structure of the ether lipids of thermophilic acidophilic bacteria of the *Caldariella* group. *Phytochemistry.* 16:1961–1965.
3. De Rosa, M., A. Gambacorta, B. Nicolaus, and J. D. Bu'Lock. 1980. Complex lipids of *Caldariella acidophila* a thermoacidophile archaebacterium. *Phytochemistry.* 19:821–825.

GLIOZZI ET AL. *Bipolar Lipid Membranes*

4. Szabo, G., G. Eisenmann, and S. Ciani. 1969. The effects of the macrotetralide antibiotics on the electrical properties of phospholipid bilayer membranes. *J. Membr. Biol.* 1:346–382.

5. Fettiplace, R., D. M. Andrews, and D. A. Haydon. 1971. The thickness, composition and structure of some lipid bilayers and natural membranes. *J. Membr. Biol.* 5:277–296.

6. White, S. H., and T. E. Thompson. 1973. Capacitance, area and thickness variations in thin lipid films. *Biochim. Biophys. Acta.* 323:7–22.

7. Benz, R., O. Frôhlich, P. Laüger, and M. Montal. 1975. Electrical capacity of black lipid films and of lipid bilayers made from monolayers. *Biochim. Biophys. Acta.* 394:323–334.

8. Requena, J., and D. A. Haydon. 1975. Van der Waals forces in oil-water systems from the study of thin lipid films. II. *Proc. Roy. Soc. Lond. A.* 47:161–177.

9. Waldbillig, R., and G. Szabo. 1978. Solvent-depleted bilayer membranes from concentrated lipid solutions. *Nature (Lond.).* 272:839–840.

10. White, S. H. 1977. A study of the physical chemistry of planar bilayer membranes using high precision measurements of specific capacitance. *Ann. N.Y. Acad. Sci.* 303:243–252.

11. White, S. H. 1978. Formation of "solvent-free" black lipid bilayer membranes from glyceryl monooleate dispersed in squalene. *Biophys. J.* 23:337–347.

12. White, S. H. 1975. Phase transitions in planar bilayer membranes. *Biophys. J.* 15:95–117.

13. Pagano, R. E., R. J. Cherry, and D. Chapman. 1973. Phase transitions and heterogeneity in lipid bilayers. *Science (Wash. D.C.).* 181:557–558.

14. Wobschall, D. 1971. Voltage dependence of bilayer membrane capacitance. *J. Colloid Interface Sci.* 40:417–423.

15. Alvarez, O., and R. Latorre. 1978. Voltage-dependent capacitance in lipid bilayers made from monolayers. *Biophys. J.* 21:1–17.

16. Montal, M., and P. Mueller. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. U.S.A.* 69:3561–3566.