J. Membrane Biol. 6, 304–314 (1971)

The Molecular Organization of Nerve Membranes

V. Properties of Mono- and Bimolecular Films Formed with Lipids Isolated from an Axolemma-Rich Preparation from Squid Retinal Axons

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Received 2 July 1971

Summary. The formation and properties of mono- and bimolecular films of total lipids extracted from an axolemma-rich preparation from retinal axons of the squid are described.

The bilayers formed with α-tocopherol showed resistance values of $10^7\ \Omega\,cm^2$ indicating their low ionic permeability. These membranes were stable for 30 to 60 min and they behave as an ohmic resistance in the range of −120 to +120 mV.

The films formed with these lipids did not discriminate between Na^+ and K^+ and showed a slight selectivity for Cl^- as compared with cations, indicating a behavior as a rather neutral barrier.

The pressure-area characteristics of monolayers built with the phospholipids fraction gave an area of 79 $Å^2$/molecule at a pressure of 10 dynes/cm. This expanded molecular area can be accounted for by the unsaturated fatty acid chains of polyenoic structure attached to these phospholipid molecules. The fraction containing 81% cholesterol presented an area of 29 $Å^2$/molecule at 10 dynes/cm.

Monolayers of the total lipids displayed an area of 51.7 $Å^2$/molecule at a pressure of 10 dynes/cm. These findings indicate that the phospholipids, when mixed with cholesterol and free fatty acids, formed rather condensated films. Cholesterol might contribute significantly to increase the cohesive forces in the film and hence to its stability.

The expanded films given by the phospholipids extracted from nerve membrane also indicate that they have a low transition temperature; their unusual unsaturated aliphatic chains might be in a special high mobile condition. Their behavior might be important for the position and order of polar groups in an excitable membrane.

Studies carried out by Fischer, Cellino, Zambrano, Zampighi, Téllez-Nagel, Marcus and Canessa-Fischer (1970) enabled the isolation of a plasma membrane preparation from retinal axons of the squid, whose axolemma to Schwann cell membrane is highly favorable to the excitable membrane.

This membrane preparation constitutes a very adequate material to study the role of the various molecular species in the process of nerve excitation.

The lipid composition of this membrane preparation has been thoroughly characterized by Zambrano, Cellino and Canessa-Fischer (1971). The fraction extracted with polar solvents constitutes 63.6% of the total lipids and contains principally phosphatidyl choline and phosphatidyl ethanolamine. The polar fraction extracted with non-polar solvents was formed by cholesterol which constitutes 22% of the total lipids, and fatty acids which constitute 5.2% ("non-polar fraction").

The various phospholipids were found to contain highly unsaturated fatty acids which may give unusual physical properties to an excitable membrane.

It seemed important to us to study the properties of these well characterized lipids forming model membranes with them as bilayers and monolayers.

A great deal of studies carried out by Mueller, Rudin, Tien and Westcott (1962), Cass and Finkelstein (1967), Lauger, Lesslauer, Marti and Richter (1967), Mueller and Rudin (1968), Szabo, Eisenman and Ciani (1969), and others, have given valuable information about electrical and selectivity properties of bilayers.

On the other hand, monolayers of lipid molecules have been used by Demel, Deenen and Pethica (1967), and Chapman, Owens, Phillips and Walker (1969) to study molecular interactions at air-water interphases.

The present work reports some of the electrical and surface properties of black membranes and monolayers formed with lipids from axonal plasma membrane.

The properties of these artificial membranes has allowed us to obtain some information about the relationship between chemical structure and function of lipids extracted from an axolemma-rich preparation.

Materials and Methods

Distilled water was passed over a mixed bed ion exchange resin and redistilled through an all-glass apparatus in the presence of $KMnO_4$. Analytical grade reagents, salts and solvents were from Merck; α-tocopherol and Tris 121 were from Sigma Chemical Co.

Preparation of Plasma Membrane Fractions

The isolation of axon membranes from the retinal nerves of the squid was carried out by using the procedure described in detail by Fischer (1970). The fraction sedimented at $100{,}000 \times g$, was lyophilized and stored at -20 °C (F-100) in vacuum dessicators.

Preparation of Lipid Extracts for Bilayers

300 to 400 mg of the lyophilized membranes in 0.25 M sucrose, 30 mM Tris-Cl, and 1.0 mM Tris-EDTA were suspended in a 6 to 8-ml mixture of chloroform/metanol (2:1) and homogenized at 4 °C with a glass-glass homogenizer. Later, it was centrifuged at 10,000 rpm for 10 min in a refrigerated Sorvall centrifuge. The supernatant was separated and taken to dryness under vacuum at room temperature.

The dry residue was reextracted twice with 2.0 ml of the chloroform/methanol mixture and, in order to eliminate insoluble material, the extract was filtered through HA millipore of 0.45 μ in pore diameter, resistant to chemical attack by the solvent mixture. The dried residue was weighted and dissolved in 1.0 ml of α-tocopherol/chloroform/methanol (5:3:2 v/v) and aliquots of 250 μliters were stored under nitrogen at −15 °C or in liquid nitrogen.

Qualitative thin-layer chromatography of the extract was carried out as described by Zambrano *et al.* (1971) as a control of the stability of the phospholipids in membrane preparation. The total lipid extract was chromatographed on thin plates of Silica gel G of 20 × 20 cm and 250 μ in thickness. From 1 to 2 mg of lipids were applied using a Hamilton syringe. The plates were developed with chloroform/methanol/water (65:25:4) and butanol/acetic acid/water (60:20:20).

Proteolipids were determined by the method described by Hess and Lewin (1965) in the lipid extract used for membrane formation.

Formation of Thin Lipid Membranes

The membranes were formed by painting the lipid solution in a 1-mm diameter hole in the side of a 5-ml polyethylene cup previously thinned. This cup was placed in a larger concentric lucite compartment of 25 ml. The inner and outer chambers were filled to the same level with 0.1 M NaCl or KCl solutions buffered with 5 mM Tris-Cl, pH 7.4. The cup was rinsed with detergent and later with glass double distilled water and chloroform/methanol (2:1 v/v). Pre-painting of the hole was carried out with the lipid solution and allowed to dry for 30 min. The temperature was controlled at 37 °C by circulating water through the bottom of the outer chamber.

The outer solution was agitated by means of a magnetic stirrer. A Harvard Dual Reciprocal Pump was used to change the ionic solution of the outer compartment, while keeping the liquid level constant to avoid membrane rupture.

The membranes were observed through a Leitz microscope to detect the interference colors and the grey sheen areas during the course of their formation.

Measurements of Electrical Properties

Two calomel/KCl electrodes connected to a 610 B Keithley electrometer were used to measure the electrical resistance of the film. The DC output of the electrometer was connected to a Varian potentiometric strip chart recorder and was used to monitor the resistance transient during the membrane formation. Two types of resistance measurements were carried out. In the one procedure, the electrometer was used in the resistance mode position. In a second procedure, the membrane voltage (V_m) was determined as a function of an external applied voltage across a known resistance of 10^9 to 10^{10} Ω (R_k) as described in Fig. 1. The current (I_c) drawn from this set up was calibrated previously. The membrane resistance (R_m) was calculated according to the equation

$$R_m = \frac{V_m}{I_c}. \tag{1}$$

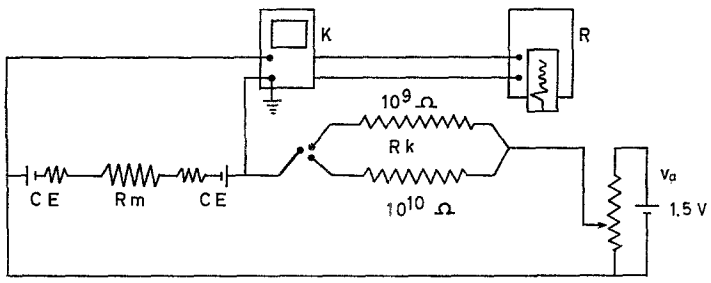

Fig. 1. Schematic diagram of the electric circuit used for membrane studies. The membrane is represented by its electrical resistance R_m. *CE* are the calomel electrodes, *K* is a 610 B Keithley electrometer, *R* is a G 11 Varian strip chart recorder. R_k are precision glass resistors

The current voltage characteristics of the membrane were obtained by means of the same circuit. A ten-turn position potentiometer connected to a 1.5 volt battery gave a variable EMF source (V_a). The electric current circulating through R_m is given by

$$I_c = \frac{V_a}{R_m + R_k}. \tag{2}$$

Since $R_k \gg R_m$ always, Eq. (2) reduces to $I_c = V_a/R_k$.

Diffusion potentials were measured with the calomel electrodes directly connected to the electrometer which was set in the voltage position.

The whole experimental apparatus was placed in a Faraday cage; all connections were made with shielded cables and UHF connectors.

Preparation of Lipid Extracts for Monolayers

Total lipid extracts were obtained by the same procedure as for bilayers, but they were solubilized in a chloroform/methanol (2:1 v/v) solution of 1.0 mg/ml.

Polar and non-polar lipid fractions were separated from these extracts by preparative thin-layer chromatography. The Silica gel G was suspended in water at approximately 30 gr/60 ml and spread by means of a Desaga applicator to obtain plates of 0.5 mm in thickness which were dried for 30 min at 110 °C. Samples of 20 mg of lipids in 1.0 ml of chloroform/methanol (2:1) were applied by a long tip pipette. The plates were developed with a mixture of petroleum ether/ethyl ether/acetic acid (85:15:1 v/v) ("non polar fraction").

Appropiate standards were run in control plates to identify the lipids which were detected with iodine vapors.

The phospholipids remained at the origin and the "non-polar" lipids were scraped off the plate. Lipids were eluted from the silica with chloroform/methanol (2:1). The eluates were centrifuged for 10 min at 10,000 rpm and filtered to eliminate the residual silica. The solvent phase was evaporated to dryness and the dry lipid weighted and dissolved in chloroform/methanol (2:1) to a concentration of 1.0 mg/ml.

The lipid solutions were flushed with N_2 and kept at -15 °C in glass bottles protected from light with aluminium foil until used.

Surface-Pressure Measurements

The surface-pressure area curves of the lipids were determined by the Wilhelmy method, using a 4-cm glass plate suspended from a torsion balance. A paraffin-coated glass trough of 62.2 cm^2 was filled with liquid subphases of 0.15 M NaCl or KCl solutions adjusted to pH 7.4 with 1.0 mM phosphate buffer.

The lipid was added by means of a 10-μliter Hamilton microsyringe in 1-μliter aliquots and each time the balance was adjusted to the equilibrium position.

Most of the experiments were performed at room temperature and lasted about 20 min.

Results

Properties of Bilayers

About 10 min after application of the lipid solution, the membrane resistance reached a constant value, between 1.6 and $3.6 \cdot 10^7$ Ωcm^2 in 15 experiments. These values are similar to those reported for other bilayers formed with synthetic or natural lipids (Hanai, Haydon & Taylor, 1965; Andreoli, Bangham & Tosteson, 1967; Lauger, 1967; Ting, Huemoeller, Lalitha, Diana & Tien, 1968; Henn & Thompson, 1969). The values obtained were similar when the membranes were formed in either KCl or NaCl solutions.

The rise in resistance was simultaneous with a change in the membrane appearance from a reflectant to a black state. Once formed, the bilayers were stable for 30 to 60 min; however, when they were formed with lipids more than a week old, they usually lasted less than 30 min. This happened even though the material was kept at −20 °C under nitrogen.

Since the lipid extract contained 3.5 μg of protein per mg of lipid, it was considered interesting to study the effect of proteolytic enzymes on the bilayer resistance. Neither pronase nor trypsin modified this electrical parameter when these enzymes were added to the solution in a concentration of 1.0 mg/ml.

Bilayers did not form at 16 °C and occasionally, but very slowly, formed at 22 °C. The rate of formation at 30 °C was about the same as that at 37 °C. Bilayers were not formed without addition of α-tocopherol.

Fig. 2 shows a typical steady state current-voltage curve for a bilayer formed with retinal axon lipids. An almost linear relationship between voltage and current is observed, meaning that films behave as an ohmic resistance between −120 and +120 mV. The linear relationship between current and voltage of the axon lipid bilayer revealed that no change in the structure of the film occurred in the ranges of potentials explored. It therefore seems that lipids alone can not account for the negative resistance characteristics of squid axon membranes.

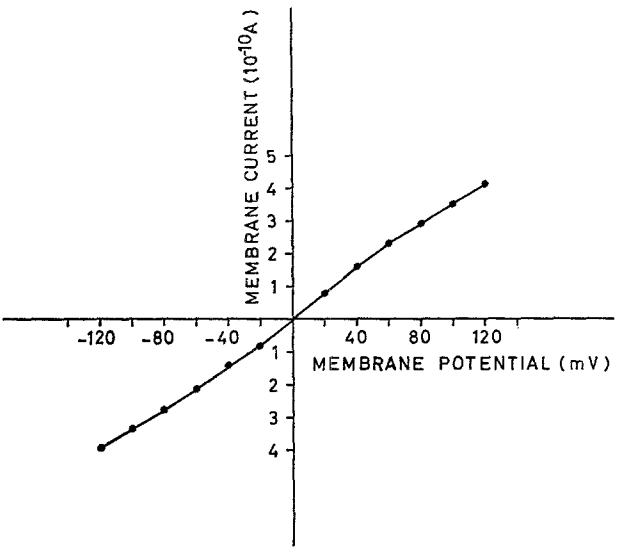

Fig. 2. Current-voltage characteristics of a typical bilayer of lipids extracted from retinal axon plasma membranes. The film was formed in an aqueous solution of 5 mM Tris-Cl, pH 7.4 and 100 mM NaCl. Temperature: 37 °C

Experiments were carried out to determine the selectivity properties of these bilayers to Na^+, K^+ and Cl^- ions. Theoretically, if a membrane separates two solutions of the same electrolyte but with different concentrations (c_1 and c_2), and assuming that electrical charges cross the membrane only in the ionic form, the following equation describes the electrical potential difference (V_m):

$$V_m=(t^-+t^+)\frac{RT}{F}\,ln\frac{(c_1\gamma_1)}{(c_2\gamma_2)_{ref}}, \tag{3}$$

where R=gas constant; F=Faraday constant; T=absolute temperature; t^+ and t^-=the transport number; and γ_1, γ_2=the activity coefficients.

Knowing that $\Sigma\, t_i=1$, and assuming that $\gamma_i=1$, the transference numbers can be calculated from these expressions. To this end, potential differences (V_m) across lipid bilayers were measured under different concentration gradients of KCl or NaCl. Table 1 shows the results of 30 such experiments.

It is seen that transference numbers are higher for anions than for cations and similar for K^+ and Na^+. It can also be seen that selectivity increases at higher concentrations. But under the experimental conditions employed in our studies, the membrane potential did not depend linealy on the log of c_1/c_2 at high ionic strength. This deviation can not be accounted for by changes in the ionic activity or osmotic effects but could be caused by a leak in the torus.

Table 1. *Ionic transference numbers in bilayers of lipids extracted from retinal axon plasma membranes*

No. of experiments	Chamber medium (inside)	Chamber medium (outside)	Mean membrane voltage	T_{Na}	T_K	T_{Cl}
5	0.1 M KCl	0.28 M KCl	5.5 mV	–	0.41	0.59
5	0.1 M KCl	0.46 M KCl	8.8 mV	–	0.40	0.60
6	0.1 M KCl	0.1 M KCl	26.0 mV	–	0.29	0.71
5	0.1 M NaCl	0.28 M NaCl	6.8 mV	0.39	–	0.61
4	0.1 M NaCl	0.46 M NaCl	10.1 mV	0.38	–	0.62
5	0.1 M NaCl	1.0 M NaCl	28.0 mV	0.28	–	0.72

Four different lipid preparations were used to form the bilayers. For each membrane, the direction of the concentration ratio was modified just once. Several membranes were made from each preparation to study the voltage dependence from the concentration ratio of KCl or NaCl.

Properties of Monolayers

These experiments were carried out with lipids obtained from three different plasma membrane preparations, no later than two days after the extracts were prepared. The surface pressure-area ($\pi - A$) characteristics of each extract was determined by running four experiments in the same day. The solutions were kept at $-20\,°C \pm 1\,°C$. This variation in temperature did not introduce any change in the results; moreover, computation of the surface pressure in four series of experiments between 18 and 24 °C did not indicate significant differences.

$\pi - A$ diagrams of total lipids isolated from plasma membranes of retinal axon, are shown in Fig. 3A. It can be seen that a surface pressure of 10 dynes/cm is exerted by $5.2 \cdot 10^3$ cm^2/mg of lipids.

Diagrams of monolayers of the "polar" and "non-polar" lipid fractions spread on similar subphases are shown in Fig. 3B. It can be observed that a surface pressure of 10 dynes/cm was displayed by $6.8 \cdot 10^3$ cm^2/mg of "polar" lipids and $4.5 \cdot 10^3$ cm^2/mg of "non-polar" lipids. These data indicate that the films of phospholipids are more expanded than those of "non-polar" fraction (i.e. cholesterol). Since the cholesterol/phospholipid molar ratio is 0.64, it can be estimated that if the mixture had an ideal behavior it must give values of $5.9 \cdot 10^3$/mg at 10 dynes/cm. However, 11 determinations carried out with total lipids gave values of $5.2 \cdot 10^3$ cm^2/mg at the same pressure indicating a condensation effect of the "non-polar fraction" upon the phospholipids.

An estimation of the mean molecular area occupied by the different lipid molecules in the film can be made on the basis of the mean molecular weight calculated from their composition as determined by Zambrano

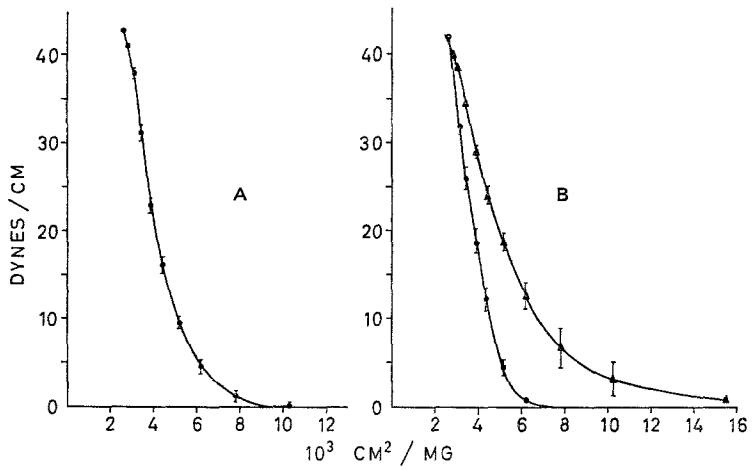

Fig. 3A and B. Surface pressure-area diagrams of monolayers of lipids extracted from retinal axon plasma membranes. The lipids were spread on a subphase of 0.15 M NaCl prepared with 1.0 mM phosphate buffer in bidistilled water at pH 7.5. (A) total lipids; (B) • non-polar lipids, ▲ polar lipids. Temperature 20 °C ± 1 °C. Each point is the average of 11 determinations

(1971) (716 for polar lipids, 387 for non-polar lipids and 595 for the total lipids, with a cholesterol/phospholipid molecular ratio of 0.64).

The molecular areas calculated at a surface pressure of 10 dynes/cm are 79 Å²/molecule of phospholipid and 29 Å²/molecule of "non-polar" lipid. The molecular area extrapolated for the total lipid extract should be 60 Å²/molecule; however, the experimental values give 51.7 Å²/molecule at 10 dynes/cm. This significant deviation from the calculated value indicates a condensation of the film most probably caused by cholesterol which constitutes 80% of the "non-polar fraction".

Monomolecular films formed with total lipid extracts stored several days without nitrogen reveal a progressive expansion in the molecular area. Five days after the lipid extraction the $\pi - A$ curve gives values as high as $8.1 \cdot 10^3$ cm²/mg at 10 dynes/cm. The changes in the surface properties of these lipids can be tentatively ascribed to the extreme reactivity to peroxidation of the polyenoic fatty acid chains which were found in these phospholipids.

Discussion

The data present indicate that bilayers formed with lipids from retinal axon plasma membranes show very high values of electrical resistance similar to those described for other phospholipid films (Huang, Wheeldon & Thompson, 1964; Andreoli *et al.*, 1967; Lauger *et al.*, 1967; and Ting *et al.*,

1968). These values of resistance are of several orders of magnitude greater than that found for the axolemma in the giant axon of the squid. The high resistance of these black membranes indicates that the solvent extraction of retinal axon membranes gives a lipid solution free from molecules as ionophores which may contribute to increase its conductance.

These lipid bilayers showed a marked unstability; they rarely lasted more than one hr. The phospholipids from retinal axon membranes have 60% of their fatty acids unsaturated, and species such as phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) were found to contain a high percentage of polyunsaturated chains. As described by O'Brien (1967), the introduction of double bonds in the chain produces a steric configuration unfavorable for the short range interactions between the fatty acid chains.

Henn and Thompson (1969) have indicated that stable bilayers are obtained when the cholesterol/phospholipid molar ratio is close to 1.0. The lipid extract from the retinal axon membranes have a cholesterol/phospholipid molar ratio lower than 1.0 (0.64). This also could explain the failure to form more stable membranes with these lipids.

However, it is also possible that the unstability of these membranes is as likely to be determined by substances present in trace amounts, as by the chemical structure of lipids extracted from the nerve membranes.

In monolayers, a remarkable increase in the molecular area was found when using aged lipid extracts. The high degree of unsaturation in the fatty acid chains may render them very sensitive to peroxidation. When forming bilayers, the addition of α-tocopherol to the extract as an antioxidant to interrupt the peroxidation reaction, was a requirement for membrane formation.

It was found that the ionic selectivity of these bilayers does not account for the behavior of intact axolemma which is more permeable to cations than to anions. This would indicate that the selective properties of the nerve membrane are not localized only in the lipid structure.

The phospholipid fraction of axon membranes contains 37% PE, 10% phosphatidyl serine (PS), 40% PC and 3.9% sphingomyelin (SM), as described by Zambrano *et al.* (1971). Therefore, the negatively charged phospholipids total 47% as opposed to 43.9% of the amphoteric PC and SM. In spite of the higher percentage of negatively charged molecules, the transference numbers measured indicated that α-tocopherol lipid bilayers behave as neutral or as slightly positively charged membranes. Bilayers formed by Andreoli *et al.* (1967) with sheep erythrocyte lipids which, according to Rouser, Nelson and Fleischer (1968), contained 24% PE, 8% PS, 19%PC and 49% SM, were cation selective even though the am-

photeric phospholipids predominated significantly. According to Henn and Thompson (1969), the effect of the polar moiety of lipids on the ionic permeability of membranes formed with a single phospholipid species and using a different solvent is not correlated to the charge of the molecule. However, recent studies of Hopfer, Lehninger and Lennarz (1970) with single lipid species concludes that the ionic selectivity was a function of the polar moiety of the molecule. Therefore, it is difficult to attempt to relate the lipid composition and the ionic selectivity in mixtures of lipids.

The surface pressure-area curves of monolayers of total lipids isolated from the retinal axons of the squid are slightly displaced towards the condensed side in comparison with results obtained by Villegas and Camejo (1968). However, they extracted total lipids from membranes isolated from giant axons of the squid which contained, according to them, mainly saturated fatty acid chains.

Monolayers formed with a mixture of total lipids were notably more packed than was expected from the cholesterol/phospholipid molar ratio of 0.64. Accordingly, it can be concluded that the "non-polar fraction" produces a condensation of the film. A great deal of studies carried out by Demel *et al.* (1967) and Chapman *et al.* (1969) have shown that unsaturated fatty acids form more expanded films than saturated ones. On the other hand, the length of the fatty acid chain produces, according to O'Brien (1967), an opposite effect. Therefore, the degree of condensation of the monolayer of phospholipids seems to reflect a balance between the high percentage of unsaturation of the fatty acids (60%) and the predominance of the fatty acid chains longer than 16 carbons. Demel *et al.* (1967) and Chapman *et al.* (1969) have also observed that the effect of cholesterol in reducing the area per molecule occupied by lecithins is much greater when cholesterol is mixed with unsaturated rather than saturated lecithins. Thus, the packing action of cholesterol fraction when it is mixed with the phospholipids as observed by us, is easily understood on the basis of these findings. This would also explain why the more saturated phospholipids of giant axon membranes isolated by Villegas and Camejo (1968) were not packed by the "non-polar" fraction.

Because phospholipids from the retinal axon membrane are highly unsaturated, they constitute an interesting material to study the role of several double bonds in their molecular interactions in experimental models of nerve membranes.

We are greatly indebted to Dr. S. Fischer for his continuous support and to Dr. G. Eisenman and Dr. A. Finkelstein for their vauable discussion. We thank Dr. F. Zambrano for his help in the lipid chemical analysis.

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