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Biochimica et Biophysica Acta 1468 (2000) 304–310

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Effect of fenitrothion on dipalmitoyl and 1-palmitoyl-2-oleoylphosphatidylcholine bilayers

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Received 10 January 2000; received in revised form 27 June 2000; accepted 29 June 2000

Abstract

The effects of the organophosphorous insecticide fenitrothion (phosphorothioic acid, *O*,*O*-dimethyl *O*-(3-methyl-4-nitrophenyl) ester; FS) on the physical state of pure dipalmitoyl (DPPC) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) membranes were investigated. FS lowers the phase transition temperature of DPPC. It has no large effects on the DPPC gel phase, but it increases the order of the liquid-crystalline state of DPPC and POPC. FS also decreases 1,6-diphenyl-1,3,5-hexatriene (DPH) lifetime (τ) in the DPPC and POPC liquid-crystalline states. Since a direct quenching of DPH emission by FS was ruled out, τ shortening is assigned to an increased water penetration in the bilayer. The effect of FS is different from most perturbing agents for which an increased order is accompanied by a higher τ. Furthermore, quenching of DPH by KI was increased by FS in POPC liposomes indicating an increased accessibility of the quencher to the hydrophobic core where DPH distributes. The effect of FS on dipole relaxation at the hydrophilic-hydrophobic interface of POPC bilayers was studied with 2-dimethylamino-6-lauroylnaphthalene (Laurdan). FS produces a decrease in Laurdan τ and a narrowing of its emission band. FS significantly increases the generalized polarization values at both emission band ends. These results indicate that FS may allow the coexistence of microdomains that have different physical properties.

Keywords: Fenitrothion; Lipid bilayers; Fluorescence quenching; Fluorescence diffusion; Lipid phase behaviour

1. Introduction

Fenitrothion (FS, phosphorothioic acid, *O*,*O*-dimethyl *O*-(3-methyl-4-nitrophenyl) ester) is an organophosphorous insecticide. Although widely used in agriculture, it has some undesirable toxic effects on non-target organisms. Because of its hydrophobicity, the compound preferentially accumulates in membrane lipids and, thus, could disturb the physicochemical and physiological properties of membranes.

Several studies have been carried out on the perturbation of the physicochemical properties of membranes by insecticides [1–7]. Antunes-Madeira et al. have reported a series of studies dealing with different insecticides and membrane models in the past

Abbreviations: FS, fenitrothion (phosphorothioic acid, *O*,*O*-dimethyl *O*-(3-methyl-4-nitrophenyl) ester); DPPC, dipalmitoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; DMSO, dimethyl sulfoxide; τ_P, phase-shift measured lifetime; τ_M, modulation lifetime; r_S, steady-state anisotropy; r_∞, limiting anisotropy; Δ, polarized phase shift

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0005-2736/00/$ – see front matter
PII: S0005-2736(00)00269-8

years [8–15]. According to reported data, some of the perturbations induced by organophosphorous insecticides depend on the cholesterol content of the membrane. High cholesterol content precludes insecticide incorporation and membrane perturbation.

Previous studies from our laboratory showed that FS increases the steady-state fluorescence anisotropy of both 1,6-diphenyl-1,3,5-hexatriene (DPH), which probes the bilayer core, and its propionic acid derivative (DPH-PA), which probes the outer regions of the bilayer. Those studies were carried out in crustacean microsomal native membranes and in liposomes made from the microsomal lipids. Phase fluorometry showed that FS does not alter the DPH rotational rate, but increases the lipid order [16].

The aim of the present study was to determine the influence of FS on single lipid bilayers of dipalmitoylphosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) to elucidate the effects of FS on biological membranes.

2. Materials and methods

2.1. Materials

DPPC and POPC were obtained from Avanti Polar Lipids (Birmingham, AL). DPH and Laurdan were purchased from Molecular Probes (Eugene, OR). Solvents were HPLC grade and were deoxygenated by sonication before use. Dipalmitoyl-[1-^{14}C]-phosphatidylcholine (113.4 mCi/mmol) was provided by New England Nuclear (Boston, MA).

2.2. Sample preparation

Multilamellar phospholipid vesicles were prepared by evaporating a chloroform-methanol (2:1, v/v) stock solution under nitrogen flow. The dry phospholipid was then hydrated with potassium phosphate buffer, 50 mM, pH 7.4, warmed at 55°C and vortexed for 30 s 10 times. The phospholipid final concentration was 150 μg/ml (200 μM).

2.3. Fluorescent measurements

All the measurements were made in a SLM 4800 C phase-modulation spectrofluorometer (SLM Instruments, Urbana, IL). For labeling, samples were mixed with few microliters of concentrated DMSO solutions of DPH, DPH-PA (final concentration 2 μM) and Laurdan (final concentration 1.3 μM).

The blanks were prepared in the same way as the samples, without the fluorescent probes, but adding the same volume of DMSO as a reference in order to correct for scattering and non-specific fluorescence. Samples were gently swirled at 20°C in the dark for at least 1 h, to allow the probes to equilibrate completely with the phospholipid bilayers. FS from ethanolic concentrated solutions (50 μg/μl) was added to the samples prior to equilibration.

2.4. Lifetime, steady-state and dynamic polarization measurements

Polarized phase shift (Δ), r_s, and τ were measured according to Lakowicz et al. [17,18] with modifications [19,20]. The excitation wavelength was 361 nm, and the emitted light passed through a sharp cutoff filter (Schott KV 389) to eliminate the light of wavelengths below 389 nm.

Measurements of τ were obtained with the exciting light amplitude-modulated at 18 and 30 MHz by a Debye-Sears modulator and vertically polarized by a Glan-Thompson polarizer. The emission light passed through the filter and then through a second polarizer oriented 55°, relative to the vertical, to eliminate effects of Brownian motion [21]. The phase shift and demodulation of the emitted light relative to a reference of known τ were determined and used to compute the phase lifetime (τ_P) and the modulation lifetime (τ_M) of the sample [22]. POPOP (1,4-bis-(5-phenyloxazol-2-yl)benzene) in ethanol, which has a τ of 1.35 ns [18,23], was used as reference. The differential polarized phase shift (Δ) was determined according to Lakowicz [17,18] by exciting with light modulated at 18 and 30 MHz and vertically polarized, and by measuring the phase difference between the parallel and perpendicular components of the emitted light.

The measured values of r_s, τ and Δ, and the fundamental anisotropy (r_0) which had been previously estimated to be 0.390 [24], were used to calculate the limiting anisotropy (r_∞) and the rotational rate as previously described [19,20] in accordance with the theory developed by Weber [25].

2.5. Laurdan generalized polarization spectra

Laurdan excitation spectra were measured at an emission wavelength of 440 nm, and the emission spectra were measured at an excitation wavelength of 340 nm. Laurdan spectral shifts were evaluated using the generalized polarization (GP) concept defined as $GP=(I_B-I_R)/(I_B+I_R)$, where I_B and I_R are the intensities observed at the blue and red maxima, respectively. For the calculation of excitation GP (exGP), intensities at the emission wavelengths 435 and 490 nm were chosen. To calculate the emission GP (emGP), the intensities for the excitation at 410 and 340 nm, corresponding to the edges of the two excitation bands, were chosen.

Laurdan fluorescence lifetime was measured as indicated for DPH by exciting at 340 nm and isolating the total emission band with a cutoff filter (KV 389) which omits light below 389 nm.

2.6. Potassium iodide quenching measurements

F_0/F ratios were plotted versus KI concentration, where F_0 and F were the fluorescence intensities of DPH (excitation wavelength 361 nm, emission wavelength 451 nm) in the absence and in the presence of KI, respectively.

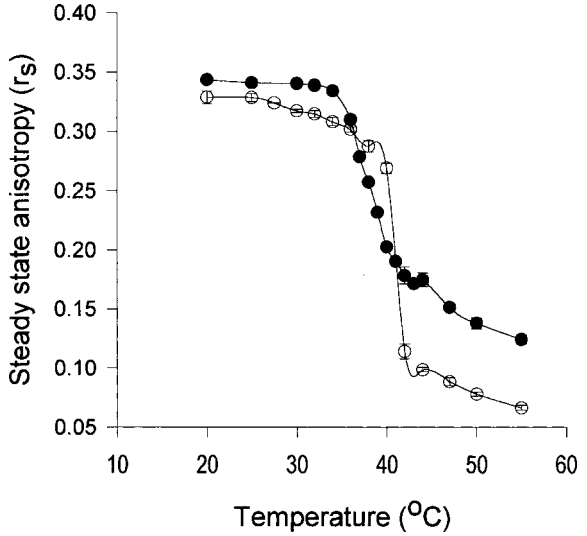

Fig. 1. Temperature dependence of the steady-state anisotropy of DPH in liposomes of pure DPPC (open symbols) and DPPC+FS 5 ppm (solid symbols).

2.7. Partition of FS into the multilamellar vesicles

The concentration of FS present in the phospholipid bilayers at 5 ppm initial buffer FS concentration was determined. The multilamellar vesicles were prepared as described earlier, but they contained 1% of ^{14}C-labeled DPPC. After exposure to FS and shaking, the liposomes were pelleted by centrifugation at $140\,000\times g$ for 2 h in a Beckman LM 10 ultracentrifuge. The phospholipid concentration was determined by liquid scintillation counting, and the FS concentration was determined spectrophotometrically at 280 nm.

3. Results

3.1. Effect of FS on the gel to liquid-crystalline phase transition of DPPC multilamellar liposomes

The effect of FS (5 ppm, 0.18 μM) on the temperature dependence of steady-state anisotropy of DPH embedded into DPPC bilayers is shown in Fig. 1. The fluorescence anisotropy of DPH significantly increases in the presence of FS in the liquid-crystalline phase of DPPC, and it is not significantly altered in the gel phase. A broadening of the cooperative phase transition and a decrease in the transition temperature midpoint (T_m) were also observed with FS interaction. Similar results were observed when POPC vesicles were assayed with FS (Fig. 2A), which significantly increased r_s in the liquid-crystalline phase.

3.2. Effects of FS on the fluorescence lifetimes (τ) of DPH in DPPC and POPC multilamellar liposomes

DPH τ is very sensitive to the polarity of the membrane environment. This probe has a low quantum yield and a very short τ when exposed to water. Thus, its τ is sensitive to the amount of water which penetrates into the lipid bilayer. Moreover, since the r_s changes can be due to variations in the rotational diffusion and/or τ variations, τ measurements are needed to correctly interpret the r_s changes caused by FS.

Fig. 2B shows the influence of FS on τ of DPH in DPPC liposomes measured at 20°C and 50°C, below

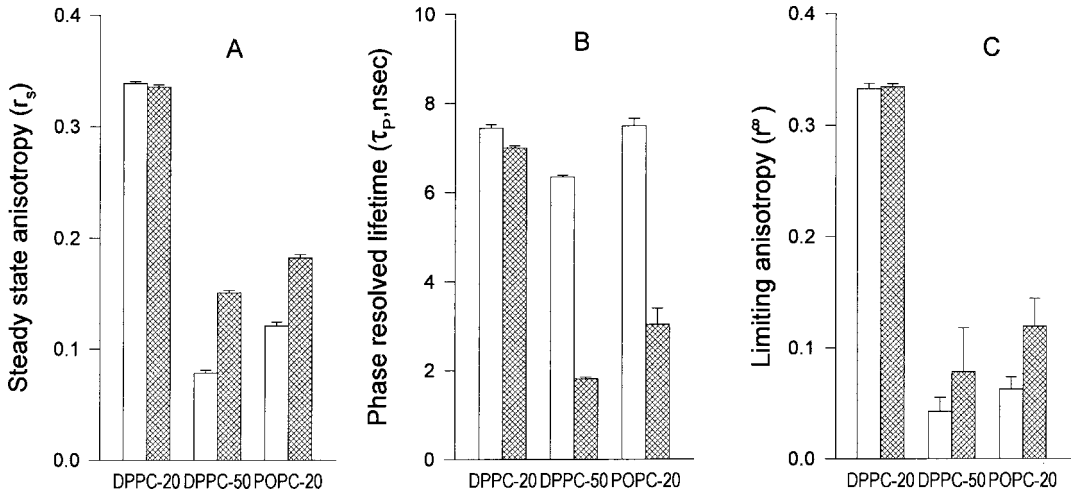

Fig. 2. Steady-state anisotropy (A), phase measured lifetime (B) and limiting anisotropy (C) of DPH fluorescence measured in the absence (empty bars) and presence (cross-hatched bars) of FS 5 ppm, in liposomes of pure DPPC at 20 and 50°C and in liposomes of pure POPC at 20°C.

and above the phase transition temperature of DPPC, and at 20°C in POPC liposomes. Phase (τ_P) and modulation (τ_M) lifetimes were measured at two frequencies, 18 MHz and 30 MHz, but the τ values were similar. The homogeneity of the τ values indicates that only one population of fluorophores can be detected in membranes. Therefore, in Fig. 2B only the τ_P values at 18 MHz are shown. Shorter τ are found in DPPC vesicles at 50°C than at 20°C, indicating that water penetration in the lipid bilayer increases in the liquid-crystalline state as compared with the gel state. A small decrease in DPH τ_P was induced by FS in DPPC vesicles in the gel state, but a large τ_P shortening was observed in the liquid-crystalline state. The fluorescence intensity and lifetime of DPH in ethanol were not affected by a large excess of FS (10 mM). This excludes the possibility of a direct quenching of DPH fluorescence by FS, indicating that the shortening of DPH lifetime produced by FS in the lipid bilayers is a consequence of alterations in the membrane structure, that promote water penetration in the bilayer.

3.3. *Effect of FS on the ordering of DPPC and POPC bilayers*

Differential polarized phase angles were measured for DPH in DPPC and POPC liposomes in the presence and absence of FS. These data, together with r_s and τ values, were used to calculate the rotational rate and r_∞ as indicated in Section 2. No marked effects of FS were found on the rotational rate of DPH (results not shown). Nevertheless, FS influences the r_∞ of DPH (Fig. 2C). The parameter r_∞ is related to the hindrance extension of the wobbling depolarizing rotation of DPH. Thus, it depends on the ordering of the lipid bilayer. As observed in Fig. 2C, r_∞ values were higher in FS exposed liposomes in the liquid-crystalline phase (DPPC at 50°C and POPC), but this effect was not observed in liposomes in the gel phase (DPPC at 20°C).

3.4. *GP of Laurdan fluorescence*

The normalized excitation and emission spectra of Laurdan in pure POPC multilamellar vesicles at 20°C in the presence and absence of 5 ppm FS are shown in Fig. 3. The emission spectrum of Laurdan is narrowed in POPC liposomes with FS. A decrease in Laurdan τ was promoted by FS: Laurdan τ_P was 4.11 ± 0.02 ns without FS and 2.58 ± 0.02 ns with 5 ppm FS measured at 18 MHz. Similar values were obtained for 30 MHz and no significant differences were observed between τ_P and τ_M. Laurdan τ shortening indicates that the environment of the fluorophore is more polar when FS is present in the bilayers.

Laurdan spectral features have been described by

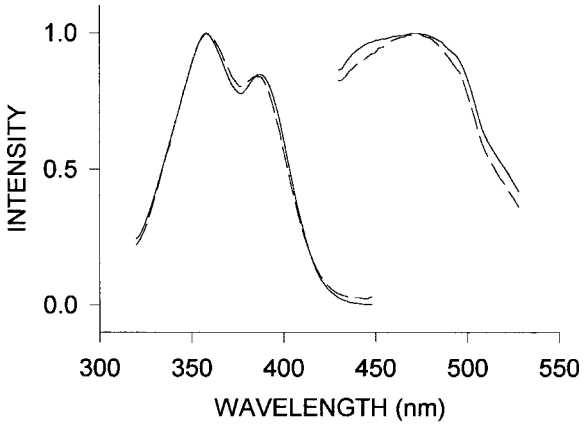

Fig. 3. Normalized excitation and emission spectra of Laurdan in pure POPC multilamellar vesicles at 20°C in the presence (dotted line) and absence (solid line) of FS 5 ppm. The excitation spectra were measured at an emission wavelength of 440 nm, and the emission spectra were measured at an excitation wavelength of 340 nm. Laurdan τ_P was 4.11 ± 0.02 ns without FS and 2.58 ± 0.02 ns with FS 5 ppm, measured at 18 MHz. Similar values were obtained for 30 MHz and τ_M.

the GP concept [26]. It has been shown that the wavelength dependence of the GP value can be used to explore the coexistence of domains of different phase states in the membrane. Excitation and emission GP spectra of Laurdan in DOPC liposomes at 20°C are shown in Fig. 4. The GP excitation spectrum is not affected by FS. In the absence of FS the GP increases with the emission wavelength as is typical of liquid-crystalline bilayers. In the presence of FS, however, the GP emission spectrum is biphasic, decreasing on the blue edge and increasing in the red of the emission band. Therefore, FS significantly increases the GP at both emission band edges.

3.5. KI quenching of DPH

Stern-Volmer plots for KI quenching of DPH incorporated in control and FS-exposed lipid bilayers in gel and liquid-crystalline phase are shown in Fig. 5. Upward deviation is evident in POPC vesicles exposed to FS, indicating that the quencher can reach the bilayer core in liquid-crystalline phase membranes. In the gel phase of DPPC vesicles, FS did not alter the KI quenching of DPH fluorescence.

3.6. Partition of FS between the aqueous phase and lipid bilayers in gel and liquid-crystalline states

In order to determine whether the limited effect of FS on the properties of lipid bilayers in the gel state could be due to low solubility of FS in phospholipids in this state, partitioning of FS between the aqueous medium and both POPC and DPPC multilamellar vesicles at 20°C was measured as described in Section 2. It was found that 85% of FS was recovered in

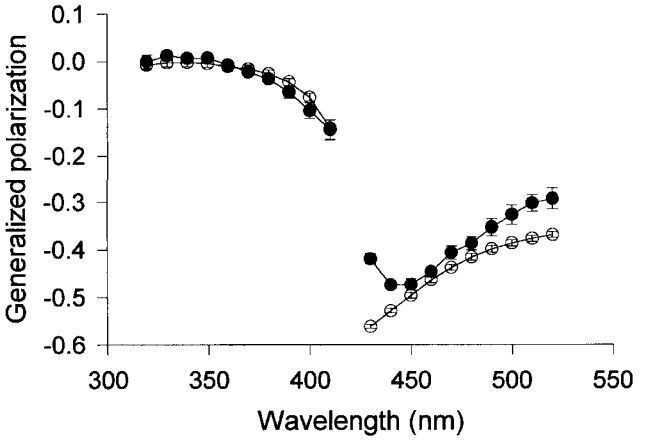

Fig. 4. Excitation and emission generalized polarization of Laurdan in pure POPC multilamellar vesicles at 20°C in the presence (solid symbols) and absence (open symbols) of FS 5 ppm. The excitation GP spectra were measured using emission wavelength of 440 and 490 nm, and the emission GP spectra were measured using excitation wavelengths of 340 and 410 nm.

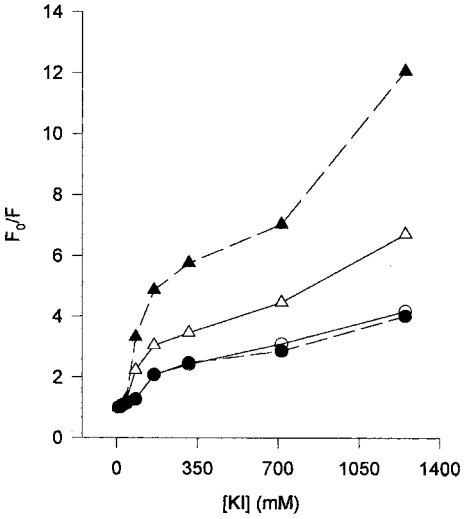

Fig. 5. Stern-Volmer plots for KI quenching of the fluorescence of DPH incorporated in DPPC (circles) and POPC (triangles) vesicles at 20°C in the presence (solid symbols) and absence (open symbols) of FS 5 ppm.

POPC as compared with 60% in DPPC multilamellar vesicles. Therefore, although FS solubility is somewhat lower in the gel state, it is not low enough to explain its limited effect.

4. Discussion

The most commonly used organophosphorous insecticides are inhibitors of acetylcholine esterase [27]. The inhibition may, in part, result from insecticide-phospholipid interactions because acetylcholine esterase is an intrinsic membrane enzyme. Several organophosphorous, chlorinated and pyrethroid insecticides have been reported to affect the fluidity of native and model membranes [1–15]. In the present work, the spectroscopic properties of different fluorescent probes were used to investigate the influence of the insecticide FS on the physical state of membrane lipids.

The temperature dependence of fluorescence anisotropy of DPH in DPPC vesicles showed that at 0.18 μM, FS lowered the transition temperature and broadened the transition width. The shifting and broadening of the transition profile may indicate the presence of foreign molecules in the cooperative region of the membrane, near the first eight carbons of the acyl chains [11,28]. In this way, FS effects are consistent with other results reported previously with different chlorinated [9–11,13,29–31], pyrethroid [2] and organophosphorous [14] insecticides. With the pyrethroid insecticide fenvalerate, although a decrease in the T_m was observed, there was no effect on the cooperativity of the transition [6].

FS did not exert rigidizing effects in the gel state bilayers, as can be seen in the r_s and the r_∞ values of DPPC vesicles at 20°C. But the rigidization of membranes was observed in the liquid-crystalline phase of both DPPC and POPC bilayers. FS partitioning at 20°C in DPPC bilayers is only 30% lower than in POPC bilayers. Thus, the lack of effect on the gel state cannot be attributed to a lower solubility of FS. The effect of FS is different from that produced by the chlorinated insecticides (DDT, DDE and lindane) or the pyrethoids which decrease fluorescence polarization in gel phases [2,6,9–11,13]. Some chlorinated compounds fluidize both gel and fluid phases [31,13]. The influence of other organophosphorous insecticides is evidenced in the liquid-crystalline phase of the bilayers, as reported for parathion [14].

The presence of FS produces a dramatic decrease in the fluorescence lifetime of DPH and Laurdan in liquid-crystalline bilayers. Since direct quenching of DPH by FS can be excluded, it is likely that FS decreases the fluorescence lifetime of these probes by generating packing defects which allow larger amounts of water to penetrate the bilayer. This is in agreement with the results of the measurements of DPH fluorescence quenching by KI, indicating that packing defects generated by FS also allow the penetration of large ions like iodide.

Whereas r_s, τ_R and r_∞ provide information about the rate of rotational behavior of the fluorophore, spectral and GP shifts of Laurdan in lipid bilayers are attributed to dipolar relaxation of the water molecules present at the hydrophilic-hydrophobic interface of the bilayer where the fluorescent naphthalene moiety of Laurdan is located [32,33]. It has been shown that increased lipid packing that results from either a liquid-crystalline to gel transition or from cholesterol incorporation into the bilayers, decreases both the water content and the dynamics of water molecules at the membrane interface, thereby causing a blue shifted Laurdan emission. The effect of FS on the Laurdan emission is atypical since no shift is evident; instead, a significant narrowing of the emission band is observed. This narrowing might be the consequence of two opposite effects. On one hand, an increased water content, indicated by the lifetime shortening, would produce a red shifted emission; on the other hand, decreased water mobility, due to a higher lipid order, would produce a blue shift. These opposite effects would result in no change in the GP values calculated between 440 and 490 nm, which are usually increased by other rigidizing agents, e.g. cholesterol.

Laurdan fluorescence can also provide information about the lateral heterogeneity of the membrane [34]. In liquid-crystalline bilayers, the GP decreases with the excitation wavelength and it increases with the emission wavelength, while the opposite behavior is attributed to the coexistence of gel and fluid phases. The presence of FS in POPC bilayers does not affect the GP dependence on the excitation wavelength, but it results in a biphasic dependence on the emission wavelength. This might indicate the presence of dif-

ferent lipid domains with different properties. However, because the Laurdan emission in FS containing bilayers is atypical, this hypothesis should be assessed by other methods.

In summary, these results show that the effects of FS on phospholipid bilayers are more evident in the biologically significant fluid state, and that the effect of FS is different from that of other perturbing agents (i.e. cholesterol), for which an increased lipid order is generally accompanied by decreased water and ion penetration in the bilayer. Thus, it is possible that the toxic properties of FS could be related to an increased permeability of membranes, a matter which should be further investigated.

Acknowledgements

This work was supported by grants from CONICET, CIC (Prov. de Buenos Aires) and UNLP, Argentina. M.R.G.B and H.A.G. are members of the Carrera del Investigador Científico, CONICET, Argentina. R.J.P. is a member of the Carrera del Investigador Científico, CIC (Prov. de Buenos Aires), Argentina. The authors wish to thank Prof. Dr. Rosalind Coleman, University of North Carolina at Chapel Hill, USA, for the revision of the manuscript.

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