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Differential effects induced by α- and β-endosulfan in lipid bilayer organization are reflected in proton permeability

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Abstract

The effects of two insecticides isomers, α- and β-endosulfan, on the passive proton permeability of large unilamellar vesicles (LUV) reconstituted with dipalmitoylphosphatidylcholine (DPPC) or mitochondrial lipids were reported. In DPPC (LUV) gel phase, at 30 °C, the global kinetic constant (*K*) of proton permeability (proportional to the proton permeability) initially increased slightly with the increase of α-endosulfan/lipid molar ratio up to 0.143. In the range from 0.143 to 0.286, a discontinuity in the increment occurred and, above this range, the proton permeability increased substantially. In DPPC fluid phase, at 48 °C, the proton permeability showed a behavior identical to that observed in gel DPPC, with a sharp increase for α-endosulfan/lipid molar ratios ranging from 0.143 to 0.286. At these and higher concentrations, α-endosulfan induced phase separation in the plane of DPPC membranes, as revealed by differential scanning calorimetry (DSC). Conversely to α-endosulfan, β-endosulfan induced only a slight increase in the proton permeability, either in the fluid or the gel phase of DPPC, for all β-endosulfan/lipid molar ratios tested. Additionally, the effects of the endosulfan isomers on the proton permeability of mitochondrial fluid lipid dispersions, at 37 °C, are similar to those described for DPPC. The β-isomer induced a very small effect, and α-endosulfan, at low concentrations, increased slightly the proton permeability, but for insecticide/lipid molar ratios above 0.143 the permeability increased substantially. Consequently, the membrane physical state of synthetic and native lipid dispersions, as affected by the structural features of α- and β-endosulfan, influenced the proton permeability. The effects here observed in vitro suggest that the formation of lateral membrane domains may underlay the biological activity of α-endosulfan in vivo, contributing to its higher degree of toxicity as compared with β-endosulfan.

Keywords: α-Endosulfan; β-Endosulfan; Lipid vesicle; Membrane organization; Proton permeability

1. Introduction

Chlorinated insecticides of the cyclodiene group are used extensively to control a variety of insect pests [1,2]. Unfortunately, the nonselectivity of these insecticides often promotes acute and chronic intoxications among nontarget organisms, either invertebrates or vertebrates, including humans [1–3]. Therefore, it is mandatory to identify the molecular mechanisms of toxicity for the sake of treatment of disorders and the development of insecticides with improved selectivity.

Although the precise molecular mechanisms responsible for the cyclodiene toxicity are still poorly understood, previous data support the idea that the main target for their action are the nerve cell membranes. Cyclodiene compounds affect the Ca^{++} permeability of presynaptic membranes [4,5] and the influx of Cl^- in postsynaptic membranes [2,6]. Additionally, these insecticides are potent inhibitors of Na^+, K^+-ATPase [7] and Ca^{++}, Mg^{++}-ATPase [8] essential for the transport of cations across the membranes [2]. Inhibition of Ca^{++}, Mg^{++}-ATPase in the presynaptic membranes results in the accumulation of intracellular free Ca^{++} ions promoting calcium-induced release of neurotransmitters [2]. Indeed, after intoxication with cyclodiene compounds, an excessive transmitter release has been observed [2,5]. However, the action of cyclodiene insecticides on membranes is not restricted to nerve cell membranes. The compounds also affect the bioenergetic properties of mitochondria [9–11] and affect the kinetics of the Ca^{++}-pump in sarcoplasmic reticulum [12]. Furthermore, the cyclodiene compounds increase glycerol permeability in pig erythrocytes [13] and

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the permeability to electrolytes and nonelectrolytes in model membranes [14]. Although the molecular mechanisms of toxicity, either acute or chronic, are still not understood, a strong interaction of cyclodienes with membranes occurs, an interaction favored by the lipophilic character of these compounds. Since most functions committed to cell membranes are regulated by the lateral and transversal organization of the lipid matrix [15,16], it has been suggested that a primary target of cyclodiene insecticides is the membrane lipid matrix.

Attempting to better understand the molecular interaction of cyclodiene compounds with the membrane lipid bilayer, studies have been previously performed with two similar compounds differing only in the position of some atoms, e.g. the α- and β-endosulfan [17,18]. The commercial formulation of endosulfan is a 7:3 mixture of α- and β-isomers [18], which differ in the degree of toxicity to mammals [19]. The availability of these isomers with different toxicities, being the α-isomer much more toxic to mammals [19], allows the elucidation of putative distinct effects on lipid membranes, permitting to understand the molecular basis for structure–activity relationship in terms of molecular toxicity. The perturbations of endosulfan isomers in lipid packing, at different depths in the bilayer, were probed by fluorescence polarization of 2-, 6- and 12-(9-anthroyloxy) stearic acids (2-AS, 6-AS and 12-AS) and 16-(9-anthroyloxy) palmitic acid (16-AP) [17], with the fluorescent anthroyloxy probes precisely located at different depths across the lipid bilayer [20]. Furthermore, to provide a better understanding of the interaction of α- and β-endosulfan with the lipid membranes, studies have been also performed by differential scanning calorimetry (DSC). The endosulfan isomers promote distinct effects in the lipid packing and in the thermotropic properties. Moreover, conversely to β-endosulfan, α-endosulfan promotes a new phase transition [17]. The distinct effects of α- and β-endosulfan are discussed in terms of molecular orientation and positioning within the bilayer. In the sequence of the above work, the present study reports the effects of both isomers in the proton permeability of dipalmitoylphosphatidylcholine (DPPC) and mitochondrial lipid vesicles as an attempt to search for a possible correlation between effects in membrane organization and membrane functionality, and lastly to provide novel insights to the understanding of molecular mechanisms of α- and β-endosulfan toxicities.

2. Materials and methods

2.1. Materials

The isomers α- and β-endosulfan [*C*,*C*′-(1,4,5,6,7,7-hexachloro-8,9,10-trinorborn-5-en-2,3-ylene) (dimethyl sulphite)] (Fig. 1) were obtained from Supelco, Inc., Bellefonte, PA 16823, USA. DPPC and all the other chemicals

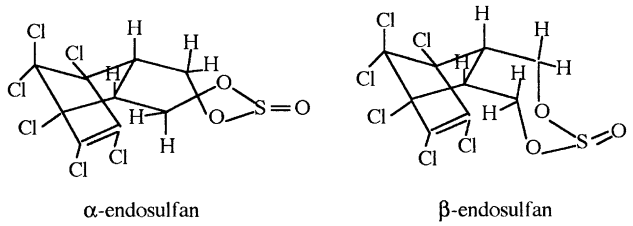

Fig. 1. Structure of α- and β-endosulfan [*C*,*C*′-(1,4,5,6,7,7-hexachloro-8,9,10-trinorborn-5-en-2,3-ylene) (dimethyl sulphite)].

were obtained from Sigma Chemical Co., St. Louis, USA. All the compounds were of the highest commercially available quality.

2.2. Preparation of membranes for proton permeability measurements

Multilamellar vesicles of DPPC and of mitochondrial lipid extracts were prepared as described elsewhere [21]. Briefly, phospholipids were dissolved in $CHCl_3$ in round bottom flasks and the solvent was evaporated to dryness. The resulting dry thin lipid film on the wall of the flask was hydrated with a suitable volume of 50 mM KCl, 5 mM Tris–maleate, 20 μM 5,6-carboxyfluorescein (5,6-CF), pH 7.4 and dispersed under N_2 atmosphere by handshaking in a water bath set 7–10 °C above the transition temperature for DPPC or at room temperature for mitochondrial lipids, and multilamellar vesicles were obtained. Large unilamellar vesicles (LUV) were prepared from multilamellar vesicles by standard extrusion techniques [22]. Thirteen repeated extrusions were performed through two stacked polycarbonate membranes of 200-nm pore size. After extrusion, unencapsulated 5,6-CF was removed by passing the liposome suspension through a 1.4×28 cm Sephadex G-50 column equilibrated with 50 mM KCl, 5 mM Tris–maleate buffer, pH 7.4. The column was maintained at 23 °C and the flow rate was 0.8 ml/min. The fraction containing DPPC (LUV) with entrapped 5,6-CF was collected. The diameters and the size distributions of vesicles were determined by dynamic light scattering using a Coulter model N4MD. The auto-correlation function was analyzed using Coulter software, applying the cumulant method and multi-exponential fit method. The average diameter of the vesicles varied between 180 and 230 nm. Phospholipid concentration in the final vesicle suspension was determined by measuring the inorganic phosphate [23] released after hydrolysis of lipid residues, at 180 °C, in 70% $HClO_4$ [24].

2.3. Proton permeability

The proton influx into the lipid vesicles, due to the action of endosulfan, is inferred from the rate of 5,6-CF fluorescence decrease, proportional to the rate of pH decrease inside the vesicles [25,26]. As the pH is lowered, 5,6-CF protonates and its fluorescence intensity decreases.

Endosulfan was added in aliquots (up to 8 μl) from concentrated ethanolic solutions (50 mM) to 2 ml of 5,6-CF-entrapped vesicle suspension, to give the insecticide/lipid molar ratios indicated in the figures. The mixture was incubated at the desired temperature for 10 min before the establishment of a pH gradient across the bilayer. Then, a pH gradient from 7.4 (inside) to 6.4 (outside) was established by adding aliquots of 0.1 M HCl. The fluorescence intensity of 5,6-CF was monitored as a function of time in a Perkin Elmer MPF-66 fluorescence spectrophotometer equipped with a thermostated cell holder. The fluorescence intensity of 5,6-CF was measured at 525 nm with excitation at 489 nm. The bandpass was 3 nm for excitation and emission beams [26]. The lipid concentration was nominally 0.2 mM in all the samples examined. Furthermore, the experiments were carried out in the presence of valinomycin (1 μg/mg lipid) and potassium ions to prevent the formation of a membrane potential due to the electrogenic flux of protons into liposomes [27]. Ethanol, at maximal concentration used (68.5 mM), had no detectable effect on the proton permeability of lipid bilayers, which is in agreement with results described by others [26]. It must be also stated that the insecticides, at the concentrations used, had no effect in the fluorescence signal of 5,6-CF.

The fluorescence intensity changes of 5,6-CF were fit to a double exponential decay function using the error minimization procedure of the KaleidaGraph software with a χ^2 between successive iterations less than 0.001%. The curve fit equation used was

$$F(t) = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$$

where A_1 and A_2 are the pre-exponential factors and k_1 and k_2 are the fast and slow rate constants, respectively. Calculating the kinetic global constant as the average rate constant $k = A_1 k_1 + A_2 k_2$ [26] and the vesicle radii determined by light-scattering methodology, the proton permeability (P) can be calculated from the following equation [28]:

$$P = kV/S = kR/3$$

where, R, V and S are the radius, volume and surface area of the vesicles, respectively.

In the present studies, the global fluorescence is controlled by the proton diffusion across the membrane and not by the K_{eq} of proton binding to carboxyfluorescein, since in a probe aqueous solution, the fluorescence decays to a minimum immediately upon the pH drop.

2.4. Statistics

Significance was calculated using Student's t-test of paired comparisons.

3. Results

The effects of α- and β-endosulfan on the passive proton permeability of LUV reconstituted with DPPC are illustrated in Fig. 2A and B. The proton influx into the lipid vesicles is measured by following the fluorescence intensity of 5,6-CF entrapped in DPPC vesicles, as a function of time. The fluorescence intensity of 5,6-CF decreases with time after a pH gradient from 6.4 (outside) to 7.4 (inside) is established. According to Massou et al. [29], this drop in pH would induce a decreasing in fluorescence of 50% and we have obtained a maximum of 45%. Therefore, a complete pH equilibrium was not reached. The decay of fluorescence

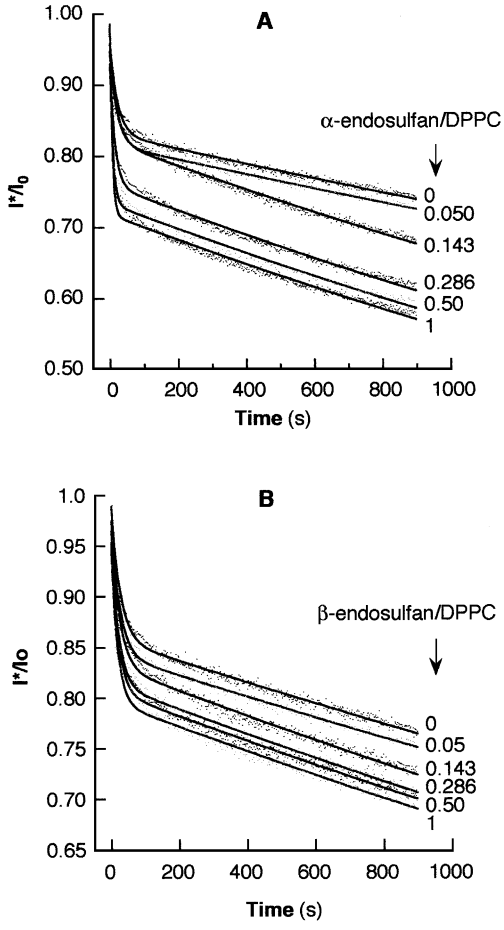

Fig. 2. Effects of increasing concentrations of α- (A) or β-endosulfan (B) on the normalized fluorescence intensity of 5,6-CF entrapped in DPPC LUV, as a function of time. The insecticide was added in aliquots (up to 8 μl) from concentrated ethanolic solutions (50 mM) to 2 ml of 5,6-CF-entrapped vesicle suspension and the mixture was incubated for 10 min, at 48 °C, before the establishment of a pH gradient, from 6.4 (outside) to 7.4 (inside), by the addition of aliquots of 0.1 M HCl. The lipid concentration was nominally 0.2 mM for all the samples examined. Valinomycin (1 μg/mg lipid) was added to prevent the development of a membrane potential due to the electrogenic flux of protons into the liposomes. The decrease in normalized fluorescence intensity reflects the influx of protons, which causes pH decrease inside the vesicles. As α- or β-endosulfan/DPPC molar ratios increase, the fluorescence intensity decreases, which reflects an increase in proton influx.

parallels the decrease of pH inside the vesicles as consequence of proton influx driven by the pH gradient [25,26]. The initial intensity is normalized to unit (normalized $F_{525\ nm} = 1$) at the point of HCl addition to elicit the pH gradient. The decrease of fluorescence intensity for α- and β-endosulfan/lipid molar ratios indicated in Fig. 2A and B follows a double-exponential decay, with a fast and a slow component, as described in Materials and methods. The double-exponential decay fits reasonably the experimental data as reflected by low χ^2 values, approaching zero, and high correlation factors (R^2), approaching the unit (Table 1). The double-exponential decay of the 5,6-CF fluorescence upon the rapid change in external pH suggests the presence of the two pools of probes. A minor pool represented by A_1 is responsible for the rapid fluorescence decay in the first seconds. The major pool represented by A_2, with slow fluorescence decay, becomes dominant only at a later period. This occurrence could be expected if a minor, but significant, portion of the probe molecules were present outside of the vesicles. However, the fast kinetic constant (K_1), increasing with insecticide concentration (Table 1), rules out this possibility. Therefore, both pools of probes are inside the vesicles. A_1 may correspond to a minor pool of molecules adsorbed to the lipid surface of the membrane, and A_2 to the water-soluble molecules. In this scenario, higher values of K_1 reflect preferential protonation of the probes adsorbed to the lipids. This also contributes the fact that proton diffusion along the lipid surface is much faster than that in the bulk water phase [30,31]. Therefore, the kinetic constant of each pool weighted by the relative proportion of probes has to be taken into account when the global kinetic constant of fluorescence decay is described.

Fig. 3A displays the effects of increasing endosulfan/lipid molar ratios on the global kinetic constant (K) of the proton permeability of DPPC (LUV) gel phase, at 30 °C, for α-(filled circles) and β-endosulfan (open circles). The global kinetic constant is proportional to the proton permeability, since the volume and surface area of the vesicles are constant, because the vesicle size remains constant. As indicated, the isomers α and β affect differently the proton permeability. Therefore, proton permeability increases only slightly for all β-endosulfan/lipid molar ratios (from 0.0 up to 1.0). For α-endosulfan, the proton permeability initially increases slightly with the increase of α-endosulfan/lipid

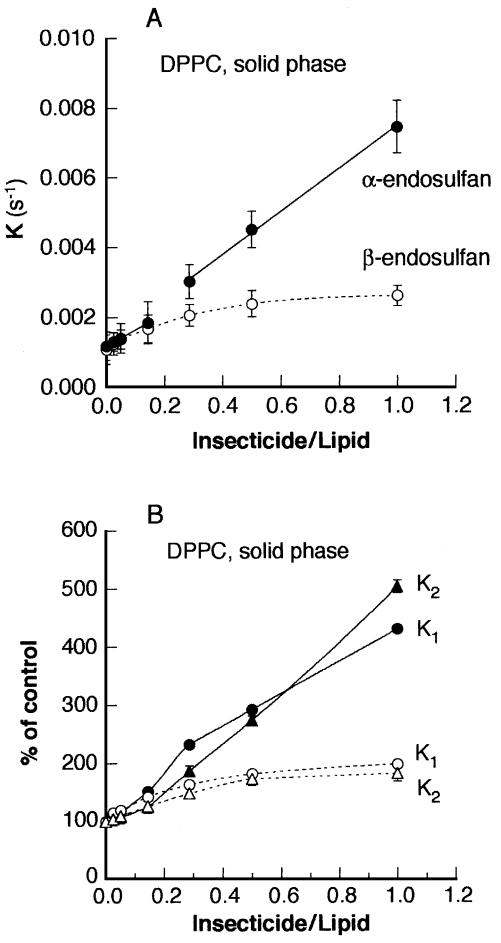

Fig. 3. (A) Effects of increasing α- (filled circles) or β- (open circles) endosulfan/lipid molar ratios on the proton permeability of DPPC (LUV) gel phase, at 30 °C, evaluated by the global kinetic constant (K). The β-isomer affects slightly the proton permeability as a function of concentration, i.e. up to 200 μM, or up to 1, in terms of insecticide/lipid molar ratio. The α-isomer initially increases slightly the proton permeability up to 0.143 insecticide/lipid molar ratio. In the range from 0.143 up to 0.286, a discontinuity in the increment of proton permeability is observed, which is more evident in B. Above this range, the increase in permeability is substantial. (B) Variation (percent of control) of fast (K_1) and slow (K_2) kinetic constants of global kinetic constant of permeability as a function of α- (filled symbols) and β- (open symbols) endosulfan/lipid molar ratios. Each point represents the mean ± standard deviation (S.D.) from three to six independent experiments. Permeability values for control samples were compared with samples treated with α- and β-endosulfan, by the Student's t-test. Only the paired comparisons for α-endosulfan show significant differences ($P < 0.0001$) for insecticide/lipid molar ratios above 0.143.

Table 1
Fluorescence intensity of 5,6-CF entrapped in DPPC

α-End/DPPC	A_1	K_1	A_2	K_2	R_2	χ^2
0	0.129	0.042	0.842	0.00013	0.979	0.0127
0.05	0.130	0.043	0.826	0.00013	0.978	0.0137
0.143	0.131	0.044	0.831	0.00016	0.985	0.0165
0.286	0.166	0.070	0.776	0.00024	0.978	0.0224
0.5	0.196	0.117	0.743	0.00029	0.974	0.0309
1	0.269	0.271	0.716	0.00037	0.981	0.0221
β-End/DPPC	A_1	K_1	A_2	K_2	R_2	χ^2
0	0.130	0.038	0.857	0.000128	0.994	0.0076
0.05	0.131	0.041	0.844	0.000130	0.990	0.0109
0.143	0.132	0.042	0.833	0.000154	0.991	0.0135
0.286	0.139	0.048	0.815	0.000157	0.989	0.0148
0.5	0.142	0.050	0.808	0.000158	0.989	0.0154
1	0.148	0.056	0.797	0.000159	0.988	0.0164

Data analysis by double-exponential decay of fluorescence intensity of 5,6-CF entrapped in DPPC LUV upon the establishment of a pH gradient from 7.4 (inside) to 6,4 (outside), at 48 °C, for several α- or β-endosulfan/DPPC molar ratios.

molar ratio up to 0.143, followed by a sharp increase at insecticide/lipid molar ratio ranging from 0.143 to 0.286. Above this range, the proton permeability increases steeply ($P<0.0001$). The abrupt increase in permeability is more apparent when the slow and fast kinetic constants are represented (Fig. 3B). The points of discontinuity occur for α-endosulfan/lipid molar ratios in the range from 0.143 to 0.286, which induce the new transition in DPPC membranes, as revealed by DSC [17]. For α-endosulfan/lipid molar ratios below 0.143, both kinetic constants are identical to those obtained with β-endosulfan, which does not induce phase separation. Worth of note is the fact that the abrupt changes in the kinetic constant of permeability indicate, according to Xiang and Anderson [32], phase separation. At α-endosulfan/lipid molar ratio of 0.143, the insecticide induces a shoulder in the main phase transition of DPPC membranes, in the low temperature side, as revealed by DSC [17]. With the increase of α-endosulfan concentration, the relative intensity of the shoulder increases and, simultaneously, the intensity of the main peak decreases. Therefore, conversely to β-endosulfan, α-endosulfan, at insecticide/lipid molar ratio of 0.143 and above, induces a new phase transition centered at 35.4 °C, in addition to the main transition [17]. In α-endosulfan/DPPC mixtures, the increasing concentration of the insecticide promotes lateral phase separation. The peak characteristic of DPPC corresponds to domains retaining a low concentration of α-endosulfan, whereas the new peak, at a lower temperature range, represents domains with a high concentration of α-endosulfan. Thermal phase diagrams for mixtures of DPPC and α-endosulfan indicate one-phase for insecticide/lipid molar ratios lower than 0.143 and two gel phases for insecticide/lipid molar ratios above 0.143, at temperatures below 33.8 °C (Fig. 4). Furthermore, between 33.8 and 40.8 °C, the diagram displays a region in which a gel phase coexists with a fluid phase. Therefore, the results of Fig. 3 are compatible with the phase diagram, since the sharp increase in the proton permeability occurs when lipid bilayers undergo a two-phase structure. The proton permeability in DPPC fluid phase at 48 °C behaves similarly as described for the gel phase. Therefore, β-endosulfan increases only slightly the proton permeability (Fig. 5A, open circles), as a function of concentration. On the other hand, the proton permeability, as affected by α-endosulfan, initially increases slightly with insecticide/lipid molar ratio up to 0.143, followed by a marked increase at insecticide/lipid molar ratio ranging from 0.143 to 0.286 (Fig. 5A, filled circles). The marked increase in proton permeability in and above this range suggests that lipid heterogeneity extends to the fluid phase in α-endosulfan/lipid mixtures. Additionally, the effects of β-endosulfan (Fig. 5A, open triangles) and α-endosulfan (Fig. 5A, filled triangles) in the proton permeability of mitochondrial lipid dispersions are similar to those described for DPPC, with a discontinuity at α-endosulfan/lipid molar ratios ranging from 0.143 to 0.286. Again, a steep increase in proton permeability is

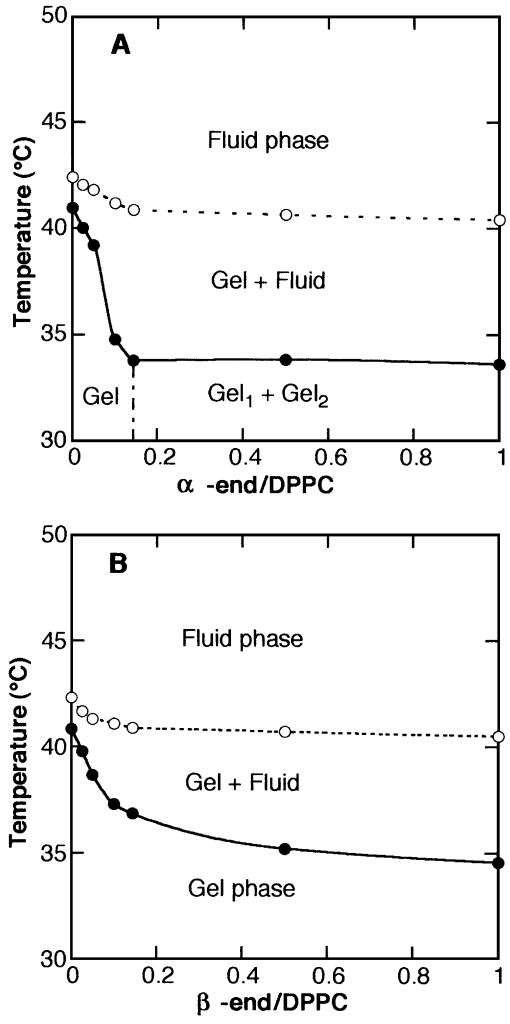

Fig. 4. Thermal phase diagrams for mixtures of DPPC and α-endosulfan (A) or DPPC and β-endosulfan (B) [17]. Black symbols and solid lines correspond to the onset temperature of the transitions and open symbols and dotted lines correspond to the completion temperature of the transitions. The vertical line (——·——) in A separates two distinct regions: (a) a domain with insecticide/lipid molar ratios lower than 0.143, which forms one phase with only one transition; (b) other domain with insecticide/lipid molar ratios higher than 0.143 and with two phases and two transitions (the main transition and the new transition).

observed ($P<0.0001$) for α-endosulfan concentrations which induce lateral phase separations in DPPC bilayers [17]. The abrupt increase in permeability induced by α-endosulfan is again more apparent when the slow and fast kinetic constants are represented (Fig. 5B).

The effective concentrations used in the present study (0–200 μM) are difficult to correlate with the toxic concentrations (LD_{50}) obtained in experimental mammals, which for α- and β-endosulfan are 76 and 240 mg/kg, respectively [33], since the distribution of insecticides in tissues depends on several parameters, including partition coefficients [34]. Both isomers have a high partition in octanol, reaching about 10^4 [35], but partitioning in biological systems is unknown. The estimation of membrane partitioning from

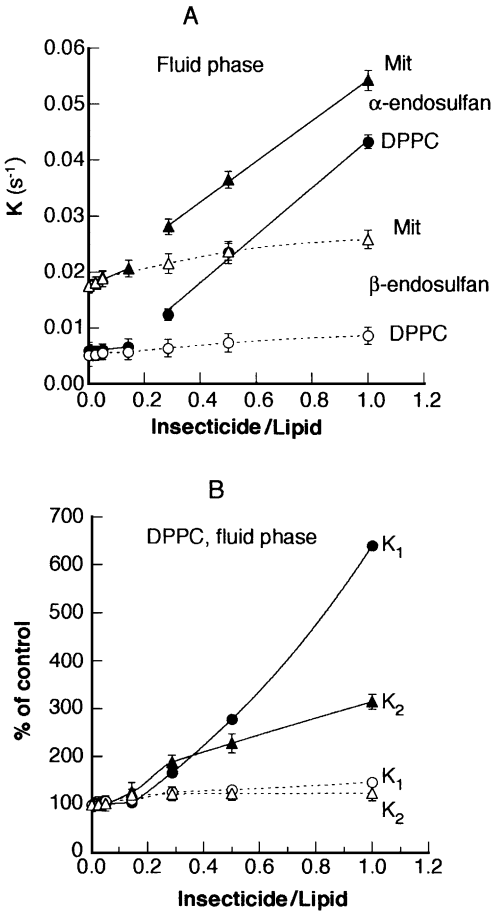

Fig. 5. (A) Proton permeability determined in DPPC (LUV) fluid phase (at 48 °C) in the presence of β-endosulfan (open circles) or α-endosulfan (filled circles). β-endosulfan increases only slightly the proton permeability, as a function of concentration, whereas α-endosulfan initially increases the proton permeability slightly with insecticide/lipid molar ratio up to 0.143, and a marked increase in permeability takes place for insecticide/lipid molar ratio ranging from 0.143 to 0.286. The effects of β-endosulfan (open triangles) and α-endosulfan (filled triangles) in the proton permeability of mitochondrial fluid lipid dispersions (at 37 °C) are similar to those observed for DPPC. Therefore, a discontinuity in the increment of permeability is observed for α-endosulfan/lipid molar ratios ranging from 0.143 to 0.286. Above this range, the increase in permeability is substantial. (B) Variation (percent of control) of fast (K_1) and slow (K_2) kinetic constants of global kinetic constant of permeability as a function of α- (filled symbols) and β- (open symbols) endosulfan/lipid molar ratios. As in the previous figure, each point represents the mean ± S.D. from three to six independent experiments. Permeability values for control samples were compared with samples treated with α- and β-endosulfan by the Student's *t*-test. The paired comparisons showed significant differences ($P < 0.0001$) for α-endosulfan/lipid molar ratios higher than 0.143 and $P < 0.1$ for β-endosulfan/lipid molar ratios ≥ 0.4.

solvent data is very rough, since the incorporation into real membranes is affected by several parameters, and mainly by the physicochemical character of membrane components and insecticide themselves [34,36,37]. Consequently, differences in insecticide localization either across the bilayer thickness or in the lateral plane of the membrane take place; therefore, differences in partition are expected. The insecticide concentrations (0–200 μM) were used on the basis of the following: significant cell death, by apoptosis, was observed in a human T-cell line for endosulfan (commercial formulation: 70% of α- and 30% of β-isomer) concentrations ranging from 10 to 200 μM [38]; the apoptotic process is related with ATP depletion caused by a drop in mitochondrial transmembrane potential due to the increase of membrane permeability; similar concentrations (up to 200 μM) of α- and β-endosulfan were here used to understand the contribution of each isomer to the proton permeability.

4. Discussion

The present study demonstrates that α- and β-endosulfan, two compounds with minor structural differences, affect differently the passive proton permeability of DPPC bilayers, either in the gel (at 30 °C) or the fluid phase (at 48 °C), as illustrated in Figs. 3 and 5. Therefore, the proton permeability increases only slightly as a function of β-endosulfan/lipid molar ratios, ranging from 0.0 up to 1.0, or in terms of insecticide concentration from 0 up to 200 μM. This slight increase in permeability may reflect an increase in the intermolecular distances of the phospholipids and, consequently, a decreased intermolecular cooperativity. Both isomers of endosulfan, at insecticide/lipid molar ratios ranging from 0.025 to 1, shift the phase transition midpoint to lower temperature values and broaden the transition profile of DPPC bilayers [17]. Furthermore, conversely to β-endosulfan, α-endosulfan, at insecticide/lipid molar ratios in the range from 0.143 to 0.286, promotes a new phase transition centered at 35.4 °C, in addition to the main phase transition of DPPC. The proton permeability, as affected by α-endosulfan, initially increases slightly with α-endosulfan/lipid molar ratio up to 0.143, followed by a discontinuity at insecticide/lipid molar ratios in the range from 0.143 to 0.286. Above this range, the proton permeability increases steeply. The critical α-endosulfan/lipid molar range, at which the sharp increase in proton permeability takes place, comes close to the α-endosulfan/lipid molar ratio that initiates phase separation in DPPC bilayers, as revealed by DSC and respective thermal phase diagrams [17]. Therefore, the sharp increase in the proton permeability probably results from the nonrandom distribution of α-endosulfan and, consequently, from the bilayer heterogeneity in terms of formation of lipid domains and associated interfacial regions [39]. The results suggest that protons can cross the membrane at the interface between the two-phases at a much faster rate than through one phase. In conditions of two-phase coexistence, occurring at the main phase transition, between gel and liquid crystalline states, a sharp increase in passive Na^+ permeability has been observed [40]. Also, the passive permeability of glucose and of small polar molecules across two-component phospholipid bilayers exhibits a maximum when gel and fluid domains coexist [41]. It has been proposed that the sharp increase in solute permeability is promoted by packing defects at the interfacial region,

hypothesis supported by computer simulation on microscopic models [42]. Accordingly, the interfaces form soft leaky regions with poor packing characteristics as they are dominated by acyl-chain conformations with some degree of disorder.

Similarly to α-endosulfan, ethylazinphos (organophosphorus insecticide) increases the proton permeability of DPPC bilayers and a sharp increase in proton permeability is observed at an insecticide/lipid molar ratio identical to that inducing phase separation in the plane of DPPC bilayers [43,44]. Furthermore, these effects of ethylazinphos are reflected in membrane potential and oxidative phosphorylation of mitochondria [44]. Also, ethanol, at concentrations which induce lateral phase separation in DPPC membranes, concomitantly enhances the proton permeability [26]. Several inhalation anaesthetics, namely, chloroform, halothane and enflurane, at high concentrations, induce lateral heterogeneities in phosphatidylcholine bilayers and, simultaneously, increase the passive permeability of carboxyfluorescein [45]. However, the relationship between the amount of interfaces and the increase in permeability is not a strict rule. For example, lindane (organochlorine insecticide) also creates lateral heterogeneities in lipid membranes [46]. However, this effect does not promote increase in permeability, but the opposite, due to a sealing effect at the interfaces [47]. Also, it has been demonstrated that low cholesterol concentrations promote an increase in the transmembrane permeability, whereas incorporation of high amounts of cholesterol decreases the permeability [48,49]. These observations are in accordance with the ability of cholesterol to increase the dynamic domain formation when present in low amounts, or to induce only one-phase, when present in the membrane at high concentrations [50,51].

On the other hand, the passive permeability phenomena can be used to predict membrane lipid heterogeneity and its modification by xenobiotic compounds [32,52]. Accordingly, the break in proton permeability in DPPC (LUV) fluid phase, for α-endosulfan/lipid molar ratios in the range from 0.143 to 0.286 as indicated in Fig. 5, may reflect regions of two-phase coexistence in the fluid phase.

Data obtained with model DPPC membranes are useful and valid but they cannot be directly extrapolated to complex native membranes. Therefore, in a first approach we have extended the above permeability studies to mitochondrial lipid dispersions. Interestingly, the effects of α- and β-endosulfan in the proton permeability of mitochondrial lipid dispersions are similar to those described for DPPC vesicles. Therefore, a marked increase in proton permeability takes place at α-endosulfan/lipid molar ratios which induce phase separations in DPPC bilayers. Consequently, the results suggest that α-endosulfan also modifies lipid organization in mitochondrial lipid membranes, creating interfaces or similar regions that promote the proton permeability.

The perturbing effects of α- and β-endosulfan in the bilayer organization and, consequently, in the proton permeability, are certainly related to the minor structural differences of the compounds [18], which dictate differences in localization across the thickness of the bilayer [17]. Accordingly, fluorescence polarization data indicate that α-endosulfan increases the lipid structural order in the regions probed by 2-AS and decreases it in the regions probed by 6-AS, 12-AS and 16-AP. On the other hand, the β-isomer produces disordering effects in the upper regions of the bilayer, probed by 2-AS, and ordering in deeper regions, probed by 6-AS, 12-AS and 16-AP. The overall data for α- and β-endosulfan suggest, as discussed previously [17], that β-isomer may incorporate more deeply in the membrane than the α-isomer. On the other hand, the shifting and broadening of the phase transition produced by both isomers [17] means, according to Jain and Wu [53], a preferential localization of the xenobiotics in the cooperativity region of the bilayer, i.e. the region of C_1–C_9 atoms of the acyl chains that determines to a large extent the sharpness of the thermal transition. Furthermore, a compound that promotes the appearance of a new phase transition extends its interactions to the polar headgroups of the phospholipids [53]. Therefore, α-endosulfan is probably located in the membrane close to the lipid–water interface with its more hydrophobic moiety buried into the cooperativity region, whereas β-endosulfan may "prefer" deeper regions of the bilayer. This localization of α-endosulfan should contribute to the promotion of phase separation, since the relationship between a preferential localization of a xenobiotic close to the lipid–water interface and the appearance of a new phase transition has been reported for several drugs [54–57].

On the basis of the above data, we propose that the minor structural differences of α- and β-endosulfan, which dictate differences in localization across the thickness of bilayer and, consequently, in membrane physical state also dictate differences in proton permeability. Additional work is under study in order to have further insights on the molecular mechanisms of α- and β-endosulfan toxicities.

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References

[1] G.T. Brooks, Action of chlorinated insecticides, in: G. Zweig (Ed.), Chlorinated Insecticides, CRC Press, Cleveland, OH, 1974, pp. 63–144.
[2] D.J. Ecobichon, Toxic effects of pesticides, in: C.D. Klaassen (Ed.), Casarett and Doull's, Toxicology, The Basic Science of Poisons, McGraw-Hill, New York, 1996, pp. 643–689.
[3] S.M. Naqui, C. Vaishnavi, Bioaccumulative potential and toxicity of endosulfan insecticide to non-target animal, Comp. Biochem. Physiol. 105C (1993) 347–364.

[4] J.M. Clark, Insecticides as a tool in probing vital receptors and enzymes in excitable membranes, Pestic. Biochem. Physiol. 57 (1997) 235–254.
[5] J.D. Doherty, Insecticides affecting ion transport, Pharmacol. Ther. 7 (1979) 123–151.
[6] I.M. Abalis, M.E. Eldefrawi, A.T. Eldefrawi, High-affinity stereospecific binding of cyclodiene insecticides and γ-aminobutyric acid receptors of rat brain, Pestic. Biochem. Physiol. 24 (1985) 95–102.
[7] L.K. Cutkomp, R.B. Koch, D. Desaiah, Inhibition of ATPases by chlorinated hydrocarbons, in: J.R. Coats (Ed.), Insecticide Mode of Action, Academic Press, New York, 1982, pp. 45–69.
[8] M. Luo, R.P. Bodnaryk, The effect of insecticides on ($Ca^{++}+Mg^{++}$)-ATPase and the ATP-dependent calcium pump in moth brain synaptosomes and synaptosome membrane vesicles from the bertha armyworm, *Mamestra configurata* Wlk, Pestic. Biochem. Physiol. 30 (1988) 155–165.
[9] R.K. Dubey, M.U. Beg, J. Singh, Effects of endosulfan and its metabolites on rat liver mitochondrial respiration and enzyme activities in vitro, Biochem. Pharmacol. 33 (1984) 3405–3410.
[10] R. Mishra, S.P. Shukla, Effects of endosulfan on bioenergetic properties of liver mitochondria from freshwater catfish *Clarias batrachus*, Pestic. Biochem. Physiol. 50 (1994) 240–246.
[11] E.M. Silva, A.M.V.M. Soares, A.J.M. Moreno, The use of the transmembrane potential, as an effective biosensor in ecotoxicological research, Chemosphere 36 (1998) 2375–2390.
[12] M.C. Antunes-Madeira, V.M.C. Madeira, Interaction of insecticides with the Ca^{++}-pump activity of sarcoplasmic reticulum, Pestic. Biochem. Physiol. 17 (1982) 185–190.
[13] M.C. Antunes-Madeira, A.P. Carvalho, V.M.C. Madeira, Interaction of insecticides with erythrocyte membranes, Pestic. Biochem. Physiol. 15 (1981) 79–89.
[14] M.C. Antunes-Madeira, V.M.C. Madeira, Interaction of insecticides with lipid membranes, Biochim. Biophys. Acta 550 (1979) 384–392.
[15] A.G. Lee, Lipids and their effects on membrane proteins: evidence against a role for fluidity, Prog. Lipid Res. 30 (1991) 323–348.
[16] J.F. Tocanne, L. Cézanne, A. Lopez, B. Piknova, V. Schram, J.F. Tournier, M. Welby, Lipid domains and lipid/protein interactions in biological membranes, Chem. Phys. Lipids 73 (1994) 139–158.
[17] R.A. Videira, M.C. Antunes-Madeira, V.M.C. Madeira, Perturbations induced by α- and β-endosulfan in lipid membranes: a DSC and fluorescence polarization study, Biochim. Biophys. Acta 1419 (1999) 151–153.
[18] W.F. Schmidt, C.J. Hapeman, J.C. Fettinger, C.P. Rice, S. Bilboulian, D. Coulson, L. McGillivray, B. Pauli, Structure and asymmetry in the isomeric conversion of β- to α-endosulfan, J. Agric. Food Chem. 45 (1997) 1023–1026.
[19] G.F. Antonious, M.E. Byers, J.C. Snyder, Residues and fate of endosulfan on field grown pepper and tomato, Pestic. Sci. 54 (1998) 61–67.
[20] K.R. Thulborn, W.H. Sawyer, Properties and the locations of a set of fluorescent probes sensitive to the fluidity gradient of the lipid bilayer, Biochim. Biophys. Acta 511 (1978) 125–140.
[21] M.C. Antunes-Madeira, V.M.C. Madeira, Effects of DDE on the fluidity of model and native membranes: implications for the mechanisms of toxicity, Biochim. Biophys. Acta 1149 (1993) 86–92.
[22] L.D. Mayer, M.J. Hope, P.R. Cullis, Vesicles of variable sizes produced by a rapid extrusion procedure, Biochim. Biophys. Acta 858 (1986) 161–168.
[23] G.R. Bartlett, Phosphorus assay in column chromatography, J. Biol. Chem. 234 (1959) 466–468.
[24] C.J.F. Böttcher, C.M. Van Gent, C. Pires, A rapid and sensitive submicro phosphorus determination, Anal. Chim. Acta 24 (1961) 203–204.
[25] F.C. Szoka, K. Jacobson, D. Papahadjopoulos, The use of aqueous space markers to determine the mechanism of interaction between phospholipid vesicles and cells, Biochim. Biophys. Acta 551 (1979) 295–303.
[26] J. Zeng, K.E. Smith, P.L.G. Chong, Effects of alcohol-induced lipid interdigitation on proton permeability in L-α-dipalmitoylphosphatidylcholine vesicles, Biophys. J. 65 (1993) 1404–1414.
[27] G.L. Barchfeld, D.W. Deamer, The effect of general anesthetics on the proton and potassium permeabilities of liposomes, Biochim. Biophys. Acta 819 (1985) 161–169.
[28] K. Elamrani, A. Blume, Effects of the lipid phase transition on the kinetics of H^+/OH^- diffusion across phosphatidic acid bilayers, Biochim. Biophys. Acta 727 (1983) 22–30.
[29] S. Massou, R. Albigot, M. Prats, Carboxyfluorescein fluorescence experiments, Biochem. Educ. 28 (2000) 171–177.
[30] J. Teissié, M. Prats, P. Soucaille, J.F. Tocanne, Evidence for conduction of protons along the interface between water and polar lipid monolayer, Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 3215–3221.
[31] M. Prats, J. Teissié, J.F. Tocanne, Lateral protons conduction at lipid–water interfaces and its applications for the chemiosmotic-coupling hypothesis, Nature 322 (1986) 756–758.
[32] T.X. Xiang, B.D. Anderson, Phase structures of binary lipid bilayers as revealed by permeability of small molecules, Biochim. Biophys. Acta 1370 (1998) 64–76.
[33] RTECS, Registry of Toxic Effects of Chemical Substances, NIOSH, US Department of Health and Human Services, Cincinnati, OH, 1997.
[34] M.C. Antunes-Madeira, V.M.C. Madeira, Membrane partitioning of organophosphorus and organochlorine insecticides and its implications for mechanisms of toxicity, Pestic. Sci. 26 (1989) 167–169.
[35] C. Hansch, A. Leo, D. Hoekman (Eds.), Exploring QSAR: Hydrophobic, Electronic, and Steric Constants, American Chemical Society, Washington, DC, 1995, p. 50.
[36] R.A. Videira, M.C. Antunes-Madeira, J.B.A. Costódio, V.M.C. Madeira, Partition of DDE in synthetic and native membranes determined by ultraviolet derivative spectroscopy, Biochim. Biophys. Acta 1238 (1995) 28–32.
[37] R.A. Videira, M.C. Antunes-Madeira, V.M.C. Madeira, Interaction of ethylazinphos with the physical organization of model and native membranes, Biochim. Biophys. Acta 1281 (1996) 65–72.
[38] K. Kannan, R.F. Holcombe, S.K. Jain, X. Alvarez-Hermandez, R. Chervenak, R.E. Wolf, J. Glass, Evidence for the induction of apoptosis by endosulfan in a human T-cell leukemic line, Mol. Cell. Biochem. 205 (2000) 53–66.
[39] O.G. Mouritsen, K. Jørgensen, Dynamical order and disorder in lipid bilayers, Chem. Phys. Lipids 73 (1994) 3–25.
[40] D. Papahadjopoulos, K. Jacobsen, S. Nir, T. Isac, Phase transitions in phospholipid vesicles: fluorescence polarization and permeability measurements concerning the effects of temperature and cholesterol, Biochim. Biophys. Acta 311 (1973) 330–348.
[41] S.G. Clerc, T.E. Thompson, Permeability of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine bilayer membranes with coexisting gel and liquid–crystalline phases, Biophys. J. 68 (1995) 2333–2341.
[42] L. Cruzeiro-Hansson, O.G. Mouritsen, Passive ion permeability of lipid membranes modelled via lipid-domain interfacial area, Biochim. Biophys. Acta 944 (1988) 63–72.
[43] R.A. Videira, M.C. Antunes-Madeira, V.M.C. Madeira, Biophysical perturbations induced by ethylazinphos in lipid membranes, Chem. Phys. Lipids 97 (1999) 139–153.
[44] R.A. Videira, M.C. Antunes-Madeira, V.M.C. Madeira, Ethylazinphos interaction with membrane lipid organization induces increase of proton permeability and impairment of mitochondrial bioenergetic functions, Toxicol. Appl. Pharmacol. 175 (2001) 209–216.
[45] M. Engelke, R. Jessel, A. Wiechmann, H.A. Diehl, Effect of inhalation anaesthetics on the phase behaviour, permeability and order of phosphatidylcholine bilayers, Biophys. Chem. 67 (1997) 127–138.
[46] M.C. Sabra, K. Jørgensen, O.G. Mouritsen, Calorimetric and theoretical studies of the effects of lindane on lipid bilayers of different acyl chain length, Biochim. Biophys. Acta 1233 (1995) 89–104.
[47] M.C. Sabra, K. Jørgensen, Lindane suppresses the lipid-bilayer permeability in the main transition region, Biochim. Biophys. Acta 1282 (1996) 85–92.

[48] E. Corvera, O.G. Mouritsen, M.A. Singer, M.J. Zuckerman, The permeability and the effect of acyl-chain length for phospholipid bilayers containing cholesterol: theory and experiment, Biochim. Biophys. Acta 1107 (1992) 261–270.
[49] O.G. Mouritsen, K. Jørgensen, A new look at lipid-membrane structure in relation to drug research, Pharm. Res. 15 (1998) 1507–1509.
[50] M.R. Vist, J.H. Davis, Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: ^{2}H nuclear magnetic resonance and differential scanning calorimetry, Biochemistry 29 (1990) 451–464.
[51] T.P.W. McMullen, R.N. McElhaney, New aspects of the interaction of cholesterol with dipalmitoylphosphatidylcholine bilayers as revealed by high-sensitivity differential scanning calorimetry, Biochim. Biophys. Acta 1234 (1995) 90–98.
[52] E.E. Williams, Membrane lipids: what membrane physical properties are conserved during physicochemically induced membrane restructuring, Am. Zool. 38 (1998) 280–290.
[53] M.K. Jain, N.M. Wu, Effects of small molecules on the dipalmitoyl lecithin liposomal bilayer: III. Phase transition in lipid bilayer, J. Membr. Biol. 34 (1977) 157–201.
[54] A. Herbette, A.M. Katz, J. Sturtevant, Comparisons of the interaction of propranolol and timolol with model and biological membrane systems, Mol. Pharmacol. 24 (1983) 259–269.
[55] T. Mavromoustakos, D.P. Yang, A. Makriyannis, Effects of anaesthetic steroid alphaxalone and its inactive Δ^{16}-analog on the thermotropic properties of membrane bilayers: a model for membrane perturbation, Biochim. Biophys. Acta 1239 (1995) 257–264.
[56] F.J. Aranda, J. Villalaín, The interaction of abietic acid with phospholipid membranes, Biochim. Biophys. Acta 1327 (1997) 171–180.
[57] R.A. Videira, M.C. Antunes-Madeira, V.I.C.F. Lopes, V.M.C. Madeira, Changes induced by malathion, methylparathion and parathion on membrane lipid physicochemical properties correlate with their toxicity, Biochim. Biophys. Acta 1511 (2001) 360–368.