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Perturbations induced by α- and β-endosulfan in lipid membranes: a DSC and fluorescence polarization study

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Abstract

The interaction of α- and β-endosulfan isomers with lipid bilayers was searched by differential scanning calorimetry (DSC) and 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). Both endosulfan isomers, at insecticide/lipid molar ratios ranging from 1/40 to 1/1, shift the phase transition midpoint to lower temperature values and broaden the transition profile of dipalmitoylphosphatidylcholine (DPPC) bilayers. At insecticide/lipid molar ratios of 1/40, the isomers fully abolish the bilayer pretransition. Conversely to β-endosulfan, α-endosulfan promotes a new phase transition, centered at 35.4°C, in addition to the main phase transition of DPPC. Therefore, the α-isomer may undergo a heterogeneous distribution in separate domains in the plane of the membrane, whereas the β-isomer may undergo a homogeneous distribution. 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 bilayers, probed by 2-AS, and ordering in deeper regions, probed by 6-AS, 12-AS and 16-AP, mainly in the gel phase. The incorporation of cholesterol into DPPC bilayers progressively decreases the effects of β-isomer which are vanished at 20 mol% cholesterol. However, this and higher cholesterol concentrations did not prevent α-endosulfan membrane interaction, as revealed by DSC and fluorescence polarization. The distinct effects promoted by α- and β-endosulfan are discussed in terms of molecular orientation and positioning within the bilayer. Apparently, the α-isomer preferentially locates closer to the phospholipid headgroups whereas the β-isomer distributes in deeper domains of the bilayer.

Keywords: α-Endosulfan; β-Endosulfan; Lipid membrane; Phase diagram; Fluorescence polarization; Differential scanning calorimetry

1. Introduction

Organochlorine insecticides have been widely used in agricultural and public health for the control of insect pests [1,2]. However, the non-selectivity of these insecticides often promotes acute and chronic intoxications among non-target organisms [1–3]. Although the molecular mechanisms responsible for the toxic effects of the organochlorine insecticides are still poorly understood, previous data support the idea that the main target for their action are the cell membranes. DDT and related compounds extend the Na^+ current in axon membranes, both in vertebrates and invertebrates [2,4,5]. Lindane and compounds of the cyclodiene family affect the Ca^{2+} permeability of presynaptic membranes [2,5] and the influx of Cl^- in postsynaptic membranes [2,6]. Addi-

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tionally, these insecticides are potent inhibitors of $(Na^+ + K^+)$-ATPase [7] and $(Ca^{2+} + Mg^{2+})$-ATPase [8] essential for the transport of cations across the membranes [2]. Inhibition of $(Ca^{2+} + Mg^{2+})$-ATPase in the presynaptic membranes might lead to an increase in intracellular Ca^{2+} and increased transmitter release [2]. After intoxication with lindane and other cyclodiene compounds, an excessive transmitter release has been observed [2,5]. However, the action of organochlorine insecticides on membranes is not restricted to nerve cells. The compounds also perturb the electron flow in the respiratory chain and the oxidative phosphorylation in mitochondria [9–11] and affect the kinetics of the Ca^{2+} pump in sarcoplasmic reticulum [12]. Furthermore, the organochlorine compounds increase glycerol permeability in pig erythrocytes [13] and the permeability to electrolytes and non-electrolytes in model membranes [14]. On the other hand, the organochlorine insecticides strongly accumulate in membrane lipid domains [15,16] and change the membrane lipid organization [17–21]. Since most functions committed to cell membranes, viz. the activity of membrane-associated enzymes, are regulated by the lateral and transversal organization of the lipid matrix [22,23], it has been suggested that a primary target of organochlorine insecticides may be the membrane lipid matrix [24].

Attempting to better understand the molecular interaction of organochlorine compounds with lipid bilayers and membranes, we selected two similar compounds differing only in the position of some Cl atoms, e.g. the α- and β-endosulfan isomers. Endosulfan is included in the cyclodiene family of organochlorines and the commercial formulation is a 7:3 isomeric mixture of α- and β-isomers [25], which differ in the degree of toxicity to mammals [26]. The availability of these isomers with different toxicities [26] and different environmental fates [26] 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.

Perturbations of endosulfan isomers in lipid packing, at different depths in the bilayer, were probed by means of several fluorescent *n*-(9-anthroyloxy) fatty acids with the fluorescent anthroyloxy probes precisely located at different depths across the lipid bilayer [27]. Furthermore, lateral heterogeneities or domains promoted by α- and β-endosulfan have been investigated by differential scanning calorimetry (DSC). These complementary techniques applied to lipid membranes open clues to the putative molecular interactions in native cell membranes, contributing to the understanding of the molecular basis of chemical toxicity.

2. Materials and methods

2.1. Materials

Cholesterol and dipalmitoylphosphatidylcholine (DPPC) were obtained from Sigma (St. Louis, MO, USA). The probes 2-(9-anthroyloxy) stearic acid (2-AS), 6-(9-anthroyloxy) stearic acid (6-AS), 12-(9-anthroyloxy) stearic acid (12-AS) and 16-(9-anthroyloxy) palmitic acid (16-AP) were purchased from Molecular Probes (Eugene, OR, USA). 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 sulfite)) were obtained from Supelco (Bellefonte, PA, USA). All the reagents used were at least 99% pure.

2.2. Methods

2.2.1. Preparation of membranes for fluorescence polarization studies

Model membranes were prepared as described elsewhere [17]. Briefly, solutions of pure phospholipids in $CHCl_3$ were taken to dryness in round bottomed flasks and the solvent was evaporated in a rotatory evaporator. The resulting lipid film on the wall of the round-bottomed flask was hydrated with an appropriate volume of 50 mM KCl, 10 mM Tris-maleate (pH 7), and dispersed under N_2 atmosphere by handshaking in a water bath set 7–10°C above the transition temperature of the phospholipids and multilamellar vesicles are obtained. Phospholipid-cholesterol bilayers were obtained by adding appropriate amounts of cholesterol to the phospholipids dissolved in chloroform. In all cases, the final nominal concentration of membrane lipids was 345 μM. Model membranes were sonicated briefly and for a controlled period of time (about four bursts of 30 s each) to avoid the turbidity to decrease below 0.15 A

units at 600 nm. This procedure does not distort the transition of lipid bilayers, but disperses aggregates, facilitating the readings of fluorescence and decreasing the scattered light. It should be pointed out that the sharpness of the phase transition and the phase transition midpoint of the control phospholipids are characteristic of multilamellar vesicles [28], and, consequently, the vesicle structure has been not modified by the sonication.

2.2.2. Incorporation of the probes and endosulfan into membranes

The fluorescent probes in dimethylformamide were injected (few μl) into membrane suspensions (345 μM in total lipid) to give a final lipid/probe molar ratio of about 300/1. The mixture was initially vigorously vortexed for 10 s, and then endosulfan was added from concentrated ethanolic solutions. It was ascertained that added concentrations of the insecticide were within the solubility range. The mixture was allowed to equilibrate in the dark, to protect the probe, for a period of 18–20 h to reach equilibrium, since the insecticide has to permeate multiple lipid bilayers. Furthermore, the insecticide uptake, in pure DPPC dispersions was allowed at the phase transition of the phospholipid, at which the incorporation of several insecticides is maximal [15]. On the other hand, the uptake of endosulfan in DPPC-cholesterol dispersions was accomplished at 46°C, i.e. in fluid DPPC bilayers. The solvents by themselves (few μl) had no detectable effects upon the assays.

2.2.3. Fluorescence polarization measurements

Fluorescence spectra were recorded in a Perkin-Elmer spectrofluorometer, model MPF-66, provided with a thermostated cell holder. The excitation was set at 365 nm and the emission was detected at 450 nm. The excitation and emission bandpass were 6 and 8 nm, respectively. The temperature of the sample was checked with an accuracy of ±0.1°C, using a thermistor thermometer. The degree of fluorescence polarization (P) was calculated according to Shinitzky and Barenholz [29] from the equation:

$$P = \frac{I_{\parallel} - I_{\perp} G}{I_{\parallel} + I_{\perp} G}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the light emitted with its polarization plane parallel ($\parallel$) and perpendicular ($\perp$) to that of the exciting beam. The grating correction factor for the optical system (G) is given by the ratio of the vertically to the horizontally polarized emission components when the excitation light is polarized in the horizontal direction [30].

A high degree of polarization reflects a limited rotational diffusion of the probes and, therefore, represents a high structural order or low membrane fluidity and vice versa. The term 'fluidity' is used here as being inversely proportional to the degree of fluorescence polarization of *n*-(9-anthroyloxy) fatty acid probes and essentially reflects the rate of motion of the phospholipid acyl chains. In bulky isotropic fluids, fluidity is given by the reciprocal of the viscosity. Thus, the fluidity used in the present work is related but not identical to the physical definition of fluidity.

When data are expressed in terms of the anisotropy factor, essentially the same conclusions and relationships are achieved. To be consistent with previous articles [17–21,31–33], polarization has been preferred in this work.

All the fluorescence measurements were corrected for the contribution of light scattering by performing control experiments without fluorescent probes.

2.2.4. DSC studies

Multilamellar vesicles for DSC measurements were prepared as described above. Endosulfan was added from concentrated ethanolic solutions (50, 100 or 200 mM) to yield the insecticide/lipid molar ratios stated in the figures. The mixtures (lipid plus insecticide) were allowed to equilibrate for 18–20 h. The lipid dispersions containing the insecticide were centrifuged at 450 000 × *g*, for 45 min, at 4°C, and the wet pellets were hermetically sealed in aluminium pans and placed in a Perkin-Elmer Pyris 1 differential scanning calorimeter. The DSC measurements were performed at a scan rate of 5°C/min, over the temperature range from 20 to 60°C. This rate yielded thermograms essentially identical to those obtained at 1°C/min, meaning that thermodynamic equilibrium is maintained at the scanning rate of 5°C/min. Data acquisition and analysis were performed using the software provided by Perkin-Elmer. To check for the reproducibility of the results, three different samples were scanned for each insecticide/lipid ratio. For each sample, three heating and two cool-

ing scans were recorded. The onset and completion of the phase transition were determined from the intersections of the peak slopes with the baseline of the thermograms [34]. Cooling scans yielded thermograms very similar to the heating scans, but, and according to the literature [35], the transitions in cooling curves are shifted, by about 1°C, to lower temperatures. Therefore, due to the supercooling phenomenon, accurate thermotropic transitions are evaluated from heating curves. Consequently, heating scans have been used throughout this work.

At the end of the experiments, the aluminium pans were opened and the samples dissolved in chloroform/methanol (5/1) mixtures. Phospholipids were quantitated by measuring inorganic phosphate [36] released after hydrolysis of the extracts at 180°C in 70% $HClO_4$ [37].

3. Results

3.1. DPPC bilayers

The thermotropic phase behavior of DPPC bilayers was studied by DSC, in the absence and in the presence of increasing concentrations of α- and β-endosulfan (Fig. 1A,B). DPPC pure bilayers display the gel to gel ($L_{\beta'}-P_{\beta'}$) pretransition centered at 35°C and the gel to liquid-crystalline ($P_{\beta'}-L_{\alpha}$) main transition at 41.4°C. The pretransition is accompanied by a small transition enthalpy of about 3.6 kJ/mol, whereas the enthalpy of the main transition is about 35.5 kJ/mol. These data for the pure lipid system are in good agreement with those reported elsewhere [28,38,39]. The thermotropic pretransition of DPPC is strongly affected by low amounts of α- and β-endosulfan, being already abolished at endosulfan/lipid molar ratios of 1/40 (Fig. 1A,B). Both isomers shift the phase transition midpoint to lower temperatures and broaden the transition profile, as a function of concentration (Fig. 1A,B). Conversely to the β-isomer, the α-isomer induces a shoulder in the main peak, in the low temperature side, at insecticide/lipid molar ratio of 1/7. Increasing the α-endosulfan concentration, the relative intensity of the shoulder increases, and, simultaneously, the intensity of main peak decreases (Fig. 1A). This behavior may be assigned to a phase heterogeneity in lipid membranes caused by the presence of α-endosulfan. Although DSC data do not rule out the possibility that the new phase transition results from the formation of separate lipid structures, recent studies (manuscript in preparation) support the phase heterogeneity hypothesis. Kinetic studies of proton permeability, in large unilamellar vesicles of DPPC, indicate an abrupt permeability increase, for α-endosulfan/lipid molar ratios that also promote the new shoulder in DSC thermograms.

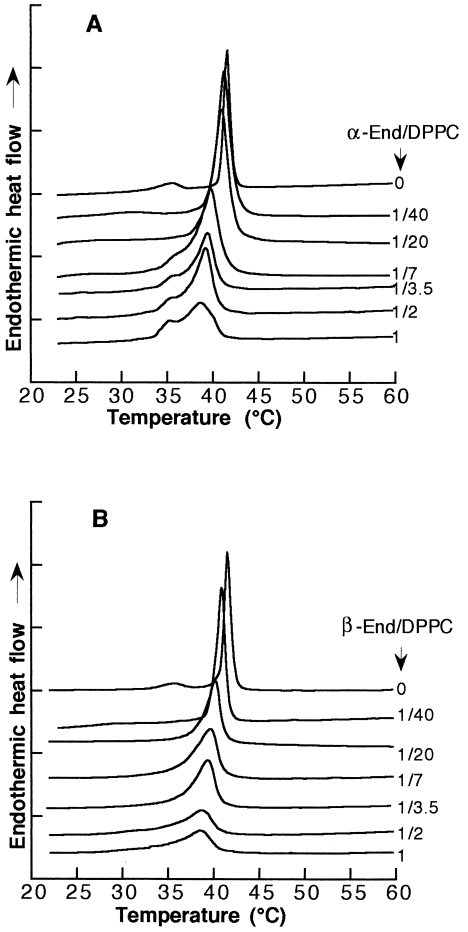

Fig. 1. DSC thermograms of DPPC bilayers in the absence and in the presence of variable concentrations of α-endosulfan (A) or β-endosulfan (B). The insecticide concentrations given in mol% with respect to lipid are indicated on the curves.

As depicted in Fig. 2, distinct effects induced by α- and β-endosulfan in DPPC bilayers are also detected in the calorimetric enthalpy (ΔH) of the thermotropic events described in Fig. 1. Therefore, concentrations of α-endosulfan up to 1/20 insecticide/lipid molar ratio promote a slight decrease of ΔH, but higher

insecticide molar fractions do not cause any further changes in ΔH (Fig. 2). Conversely, β-endosulfan strongly decreases ΔH up to a molar ratio of 0.2. Further increase of the ratio has a limited effect on ΔH.

Partial phase diagrams, which provide information regarding the equilibrium between the gel and fluid phases in the plane of the membrane, are represented in Fig. 3 for DPPC/α-endosulfan and for DPPC/β-endosulfan systems. These phase diagrams were drawn with the results obtained in DSC experiments of Fig. 1, by plotting the onset and completion temperatures of the phase transitions as a function of the insecticide/lipid molar fractions. These partial phase diagrams do not consider the pretransition of DPPC bilayers, since it is abolished at very low insecticide concentrations (Fig. 1). In α-endosulfan/DPPC systems (Fig. 3A), the temperature of the fluidus line decreases with the increase of α-endosulfan concentrations, indicating miscibility in the fluid phase. However, the decrease is steeper for α-endosulfan molar fraction up to 0.145, than for higher values. On the other hand, the temperature of the solidus line decreases sharply with insecticide/lipid molar ratio up to 0.145 and remains flat for higher ratios, indicating immiscibility in the gel phase [28]. These data indicate that a non-ideal miscibility behavior is evident for α-endosulfan/lipid molar ratios higher than 0.145 and for temperatures below 33.8°C. Therefore, in these conditions, two gel phases, gel_1 and gel_2, are apparent. Additionally, between 33.8 and 40.8°C, the diagram displays a region in which a gel phase coexists with a fluid phase. In β-endosulfan/DPPC systems (Fig. 3B), the solidus as well as the fluidus line decrease non-linearly with the increase in β-endosulfan concentrations. However, the decrease is steeper for β-endosulfan molar fraction

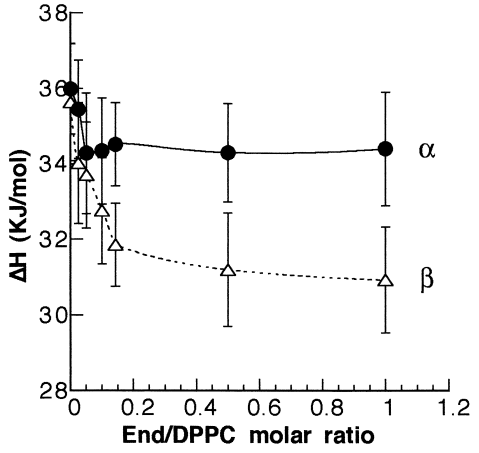

Fig. 2. Enthalpy change for the thermotropic profile of DPPC bilayers, as function of α-endosulfan (solid symbols and lines) or β-endosulfan (open symbols and dotted lines) concentrations given in mol% with respect to lipid. Each point represents the average of at least five independent measurements (vertical bars indicate ±S.D.).

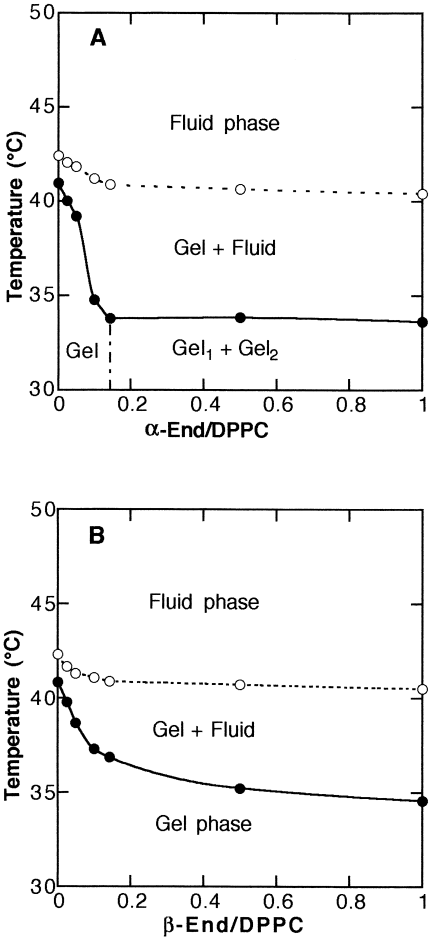

Fig. 3. Thermal phase diagrams for mixtures of DPPC and α-endosulfan (A) or DPPC and β-endosulfan (B). The diagrams were built with data from the thermograms of Fig. 1. Black symbols and solid lines correspond to the onset temperature of the transitions and open symbols and dotted lines correspond to 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 1/7, which forms one phase with only one transition; (b) a domain with insecticide/lipid molar ratios higher than 1/7 and with two phases and two transitions (the main transition and the new transition).

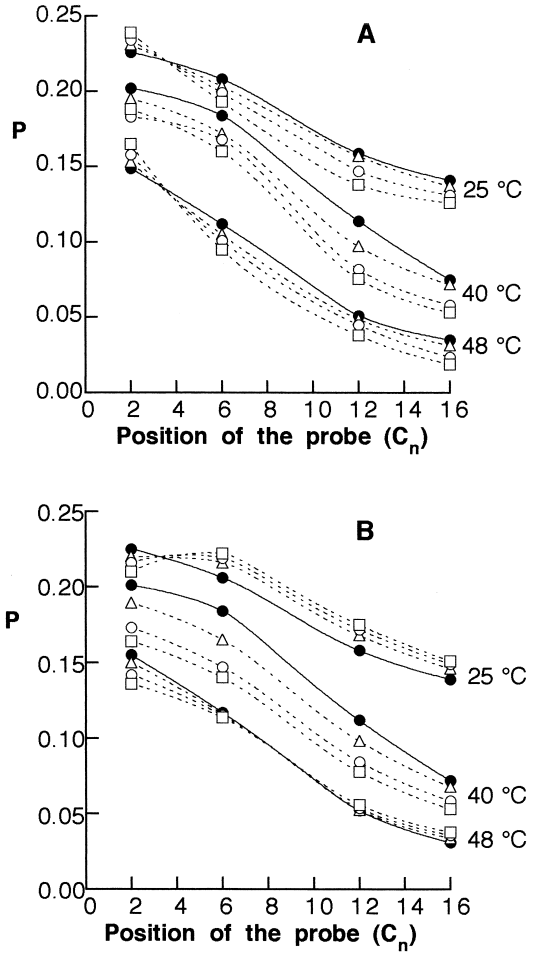

Fig. 4. Fluorescence polarization (*P*) detected by 2-, 6- and 12-AS and 16-AP, in DPPC bilayers, at 25, 40 and 48°C, in the absence (solid symbols and lines) and presence (open symbols and dotted lines) of increasing concentrations (△, 17.5 μM; ○, 50 μM; □, 100 μM) of α-endosulfan (A) or β-endosulfan (B). These concentrations correspond to insecticide:lipid stoichiometries of 1:20, 1:7 and 1:3.5, respectively. The effects of α- and β-endosulfan on DPPC bilayers were detected at 20 distinct temperatures in the range from 20 to 50°C, but only the results at the above temperatures are shown here. The standard deviations of the average of three experimental measurements, for each temperature, are too small (from 0.00197 to 0.00326) to be displayed by error bars, since for most points they are encompassed by the size of the symbols.

up to 0.145 than for higher ratios. Furthermore, the decrease in solidus line is considerably larger as compared with the fluidus line.

The effects of α- and β-endosulfan on the physical organization of DPPC bilayers have also been studied by fluorescence polarization of 2-, 6-, 12-(9-anthroyloxy) stearic acids and 16-(9-anthroyloxy) palmitic acid, i.e. 2-AS, 6-AS, 12-AS and 16-AP, respectively. These fluorescent probes, located at various depths in the membrane, report the hydrocarbon order gradient across the bilayer thickness [27], allowing to evaluate the relative perturbations of the insecticide on different segments across to the membrane. Although excited state lifetimes of the probes have not been measured, we are confident that any effect of α- and β-endosulfan on the probe lifetime is probably not significant, owing to the fact that the steady state fluorescent signal is not affected by any of these insecticides. Additionally, excitation and emission fluorescence spectra were recorded, in the absence and presence of the insecticides, to ascertain that they do not alter the relative amplitude and position of the characteristic spectral peaks (data not shown). On the other hand, the above probes, within the used concentration range (probe/lipid ratio is 1/300), have no detectable effects in the bilayer structure [40]. Local perturbations caused by the probes cannot be fully ruled out, but the contribution of these perturbations is probably small, since the reported order gradient agrees with the conclusions provided by non-perturbing techniques [41]. Therefore, the detected alterations in fluorescence polarization primarily reflect the perturbations induced by the insecticides on the rate and amplitude of motion of the phospholipid acyl chains, i.e. on the lipid packing order.

Fluorescence polarization (*P*), detected by the above probes, in DPPC bilayers, at 25, 40 and 48°C, in the absence (solid symbols and lines) and presence (open symbols and dotted lines) of increasing concentrations (△, 17.5 μM; ○, 50 μM; □, 100 μM) of α-endosulfan or β-endosulfan is represented in Fig. 4A and Fig. 4B, respectively. As shown in Fig. 4A, α-endosulfan increases the order in the region probed by 2-AS, i.e. close to the lipid-water interface, and decreases it in the regions probed by 6-AS, 12-AS and 16-AP, either in the solid (at 25°C) or in the fluid (48°C) phase. All the probes detect disordering effects at 40°C, i.e. at a temperature close to that of the main phase transition of DPPC. The β-isomer (Fig. 4B) decreases the fluorescence polarization of 2-AS and increases the fluorescence polarization of 6-AS, 12-AS and 16-AP, in the solid phase. This means that β-endosulfan decreases the lipid order parameters close to the lipid-water interface and

increases the order (decreasing of fluidity) in deeper regions of the bilayer. Furthermore, all the probes detect strong fluidizing effects at temperatures close to the main phase transition, i.e. at 40°C. In the fluid phase, the probe 2-AS detects decreasing in fluorescence polarization and the other probes detect almost no effect. This suggests that the fluid phase provides enough free volume to accommodate the insecticide without significant perturbation of general membrane organization.

3.2. DPPC-cholesterol bilayers

Fig. 5A displays the DSC profiles of DPPC bilayers enriched with increasing concentrations of cholesterol. For cholesterol concentrations lower than 20 mol%, the DSC trace of the main transition is asymmetrical when compared with the transition of the pure phospholipid (Fig. 5A). This asymmetrical transition can be deconvoluted as a sum of two symmetrical peaks, a sharp and a broad peak [42,43]. According to McMullen and coworkers [43,44], the existence of a sharp and a broad transition, at cholesterol concentrations lower than 20–25 mol%, results from the lateral phase separation of cholesterol-poor and cholesterol-rich domains, respectively. On the other hand, fluorescence polarization shows that the effects of increasing concentrations of cholesterol on the fluidity of DPPC bilayers detected by the probe 2-AS differ from those detected by the probe 16-AP (Fig. 5B). In the regions probed by 2-AS, cholesterol concentrations up to 5 mol% induce significant decrease of P, whereas higher concentrations have an opposite effect, either in the solid or in the fluid phase of DPPC. Close to the bilayer center, 16-AP detects that cholesterol concentrations up to 5 mol% increase P values, whereas increased concentrations decrease P, mainly in the solid phase (Fig. 5B). Therefore, small cholesterol concentrations induce ordering in the center of the bilayer and, simultaneously, disordering in the bilayer regions close to the lipid-water interface. These ordering and disordering effects reach a maximum at 5 mol% of cholesterol. High cholesterol concentrations ($\geq$20 mol%) induce an opposite effect, i.e. ordering in the bilayer regions close to the phospholipid headgroups and disordering in the center of the bilayer. These data strongly support the idea that the sterol at low concentrations (below 5 mol%) is preferentially located into the hydrophobic core of the bilayer, where the molecule may assemble in transbilayer dimers [45,46]. Thus, the primary effect of cholesterol is putatively related to occupancy of free volume [47], which is larger in the bilayer center [48]. Furthermore, when the cholesterol reaches a critical concentration in the membrane (about 5 mol%), it diffuses to the regions close to the lipid-water interface, packing with the phospholipid molecules in the cooperativity region of the membrane, as demonstrated by Engelman and Rothman [49]. Thus, the cholesterol-

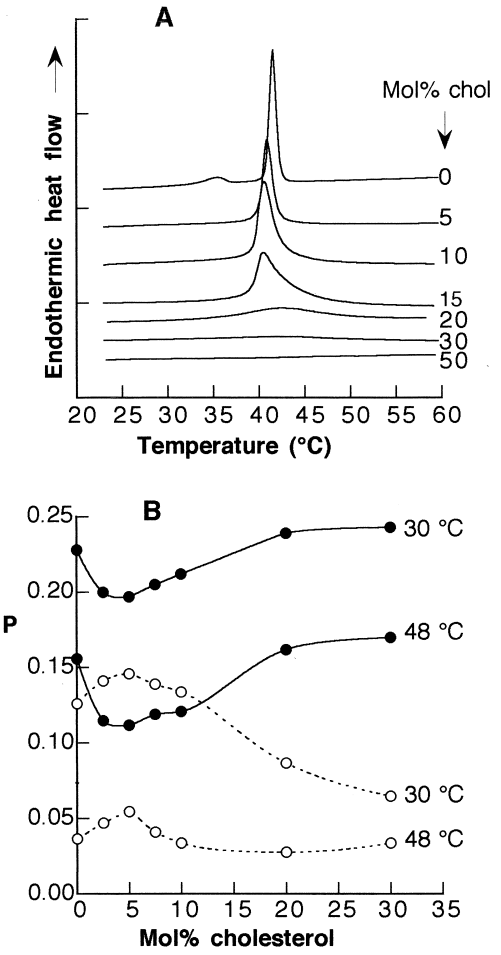

Fig. 5. (A) DSC profiles of DPPC bilayers enriched with increasing concentrations of cholesterol as a function of temperature. (B) Fluorescence polarization (P) detected by 2-AS (solid circles and lines) and 16-AP (open circles and dotted lines) in DPPC bilayers enriched with increasing concentrations of cholesterol, at 30°C (gel phase) and 48°C (fluid phase). As in Fig. 4, standard deviations of the experimental points of B were to small to be included.

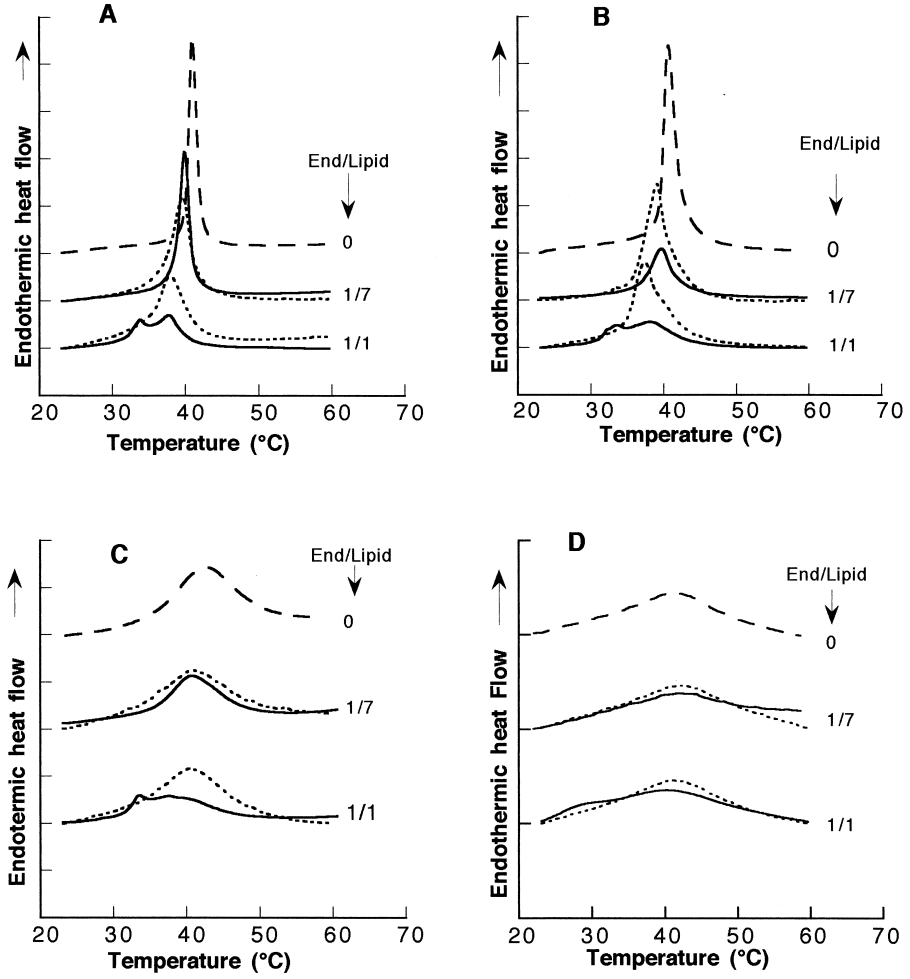

Fig. 6. DSC profiles of DPPC bilayers enriched with increasing cholesterol concentrations as a function of temperature. A, B, C and D correspond to mixtures of DPPC and 5, 10, 20 and 30 mol% cholesterol respectively, in the absence (dashed lines) and in the presence of α-endosulfan (solid lines) or β-endosulfan (dotted lines). The insecticide/lipid molar ratios are expressed on the curves. The endothermic flow values for C and D are multiplied by a factor of 2 relatively to A and B. DSC thermograms for endosulfan/lipid molar ratios of 1/40, 1/20, 1/3.5 and 1/2 were also performed, but are not shown here.

rich and cholesterol-poor domains in DPPC-cholesterol systems [44] that occur at cholesterol concentrations higher than 5 mol% and lower than 20 mol% may be connected with the diffusion of cholesterol from the bilayer center to the regions close to the lipid-water interface.

Let us now consider the effects of endosulfan isomers in DPPC-cholesterol systems (Figs. 6 and 7). In DPPC bilayers with low amounts of cholesterol ($\leq$10 mol%), where cholesterol-poor domains dominate, the effects of β-endosulfan are qualitatively identical to those described for DPPC pure systems (Fig. 6A,B, dotted lines). Thus, DSC reveals that β-endosulfan induces a decrease in the phase transition temperature midpoint and a broadening of the phase transition peak. The intensity of both effects increases with insecticide concentration. Apparently, β-endosulfan fails to perturb DPPC bilayers with high amounts of cholesterol ($\geq$20 mol%), as concluded from the DSC thermograms (Fig. 6C,D, dotted lines). Fluorescence polarization data also indicate that, at these high cholesterol concentrations, β-endosulfan does not affect the fluorescence polarization of either 2-AS or 16-AP (Fig. 7, triangles). This means that the insecticide only perturbs the cholesterol-poor domains that produce the sharp component in DSC thermograms [43,44]. Although partition data are not available, these results suggest

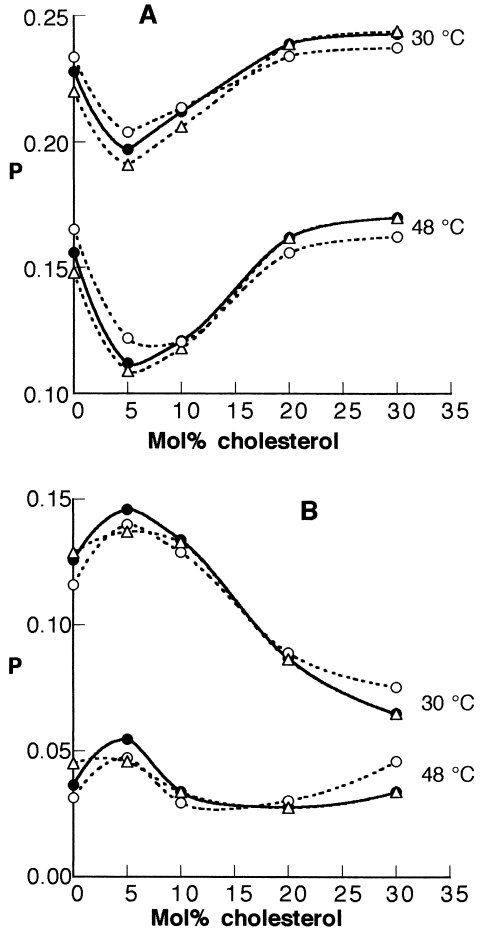

Fig. 7. Fluorescence polarization (*P*) of 2-AS (A) and 16-AP (B) as a function of cholesterol concentration, in the absence (solid symbols and lines) and in the presence (open symbols and dotted lines) of 50 μM of α-endosulfan (○) or β-endosulfan (△). Standard deviations of the experimental points were not included for the same reasons given in the legend to Fig. 4.

that DPPC/cholesterol-rich domains may exclude the insecticide from the membrane, as described for the organochlorine insecticide lindane [50] and for some organophosphorus insecticides, namely parathion, malathion [15] and azinphos [32].

Calorimetric studies in DPPC-cholesterol bilayers show that α-endosulfan, for insecticide/lipid molar ratios of 1/7, induces a broadening of the thermotropic profile and shifts the phase transition midpoint to lower temperatures (Fig. 6, solid lines). Additionally, and according to the results in DPPC pure bilayers at insecticide/lipid molar ratios higher than 1/7, a new phase transition appears, with the peak centered at about 34°C. Furthermore, the intensity of the new peak decreases slightly with the increase of cholesterol up to 10 mol% and strongly for higher cholesterol concentrations. It should be pointed out that the endothermic flow values for parts C and D of Fig. 6 are multiplied by a factor of 2 relatively to parts A and B. Therefore, the real heights of peaks in C and D are half of the represented draws. However, in bilayers with 30 mol% cholesterol a small shoulder centered at about 30°C is still apparent. Fluorescence polarization data indicate that, at α-endosulfan/lipid molar ratios of 1/7, the probe 2-AS detects ordering effects in membranes with cholesterol concentrations up to 10 mol% and disordering for higher cholesterol concentrations, either in the solid or in the fluid phase (Fig. 7A, open circles and dotted lines). In contrast, the probe 16-AP detects disordering effects in membranes with 5 and 10 mol% of cholesterol and ordering in membranes with 20 and 30 mol% of cholesterol, either in the solid or in the fluid phase (Fig. 7B). Therefore, both probes indicate that there is a critical cholesterol concentration which determines a crossover in α-endosulfan effects from ordering to disordering in the upper regions of bilayer and from disordering to ordering in the hydrophobic core.

4. Discussion

According to DSC data, endosulfan isomers induce distinct concentration dependent effects on the thermotropic profiles of DPPC bilayers (Fig. 1). The lipid perturbations induced by α- and β-endosulfan share certain properties and differ in others. At low concentrations, α- and β-endosulfan abolish the pretransition of DPPC bilayers. Additionally, they displace, progressively, the phase transition midpoint to lower temperatures and broaden the transition profile of DPPC bilayers (Fig. 1), meaning that the size of the cooperative unit undergoing transition decreases with endosulfan concentration. Furthermore, the broadening also indicates that both isomers expand the temperature range of density fluctuations, resulting from dynamic lateral bilayer heterogeneity, in terms of formation of lipid domains and associated interfacial regions [51]. Conversely to β-endosulfan, α-endosulfan, at insecticide/lipid molar ratios of 1/7 and higher, induces a new phase transition

centered at 35.4°C, in addition to the main phase transition (Fig. 1). Therefore, in α-endosulfan/DPPC mixtures, the increasing concentration of the insecticide promotes lateral phase separation (Fig. 3) due to the non-random distribution of the insecticide. 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.

According to Jain and Wu [52], a shifting and broadening of the phase transition means a preferential localization of the xenobiotic 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 [52]. Therefore, α-endosulfan is probably located in the membrane close the lipid-water interface with its more hydrophobic moiety buried into the cooperativity region, whereas β-endosulfan may prefer deeper regions of the bilayer. The relationship between a preferentially localization of a xenobiotic close to the lipid-water interface and the appearance of a new phase transition in PC bilayers has been reported for several drugs [53–55].

The fluorescence polarization of *n*-(9-anthroyloxy) fatty acids (Fig. 4) reports that the dynamics of DPPC bilayers is differently affected by α- or β-endosulfan. In turn, the physical state of the lipid modulates the effects of the isomers. The α-isomer perturbs the packing order across all the thickness of the membrane, either in the solid or in the fluid phase. However, it increases the order close to the polar headgroups, as evaluated by 2-AS, and, simultaneously, decreases it in regions probed by 6-AS, 12-AS and 16-AP. On the other hand, the β-isomer decreases the lipid packing in regions close to the polar surface, either in the solid or in the fluid phase and increases the order in deeper regions of the bilayer, as evaluated by 6-AS, 12-AS and 16-AP. This ordering effect is more apparent in the gel phase. Both isomers induce strong disordering effects across all the thickness of the bilayer in the phase transition temperature range (Fig. 4). The overall data for α- and β-isomers of the endosulfan suggest different locations across the thickness of the bilayer. Therefore, the β-isomer may incorporate more deeply into the membrane than α-isomer. The incorporation of β-endosulfan into deeper regions of the bilayer would induce expansion of the phospholipid headgroup region, which results in an increase of the free volume available for rotation of the probe 2-AS. Simultaneously, the rotational motion of the probes 6-AS, 12-AS and 16-AP decreases due to an increase of the hydrocarbon chain packing order in the region probed by these probes. On the other hand, α-endosulfan may be preferentially located close to the regions probed by 2-AS, i.e. near the lipid-water interface. The shorter molecule of endosulfan, as compared with phospholipids, may create a void in deeper regions of the bilayer, which can be filled by the acyl chains of the phospholipids. Consequently, the insecticide increases the freedom of motion of lipid hydrocarbon chains (*trans-gauche* isomerization) in these regions, as detected by 6-AS, 12-AS and 16-AP. Therefore, the effects of α-endosulfan on DPPC bilayers may result from the hydrophobic mismatch between the effective length of the insecticide molecule as compared to that of DPPC hydrocarbon chains.

The structural differences between α- and β-endosulfan also support the localization suggested above for the two isomers. X-Ray and NMR studies [56] provide unequivocal proof that the β-isomer is symmetrical, whereas the α-isomer is a mixture of two structurally indistinguishable asymmetrical molecules. This means that the α-isomer is a molecule with an electrical dipole (polar), whereas the β-isomer is fully apolar.

Data of Fig. 5 are consistent with the idea that cholesterol, at low concentrations, populates preferentially the hydrophobic core of the bilayer. Thus, the primary effect of cholesterol is putatively related to occupancy of free volume, which is larger in the bilayer center [48]. Furthermore, when cholesterol concentration increases, the molecule diffuses to the regions close to the lipid-water interface, packing with phospholipid chains in each leaflet of the membrane [49]. This organization of cholesterol modulates to a large extent the interaction of endosulfan with lipid bilayers. Therefore, β-endosulfan perturbs DPPC systems with low amounts of cholesterol, as shown by DSC and fluorescence polarization (Figs. 6

and 7). Above 10 mol% cholesterol, the effects promoted by the β-isomer weaken and practically vanish at 20 mol%. Thus, the membrane organization induced by high cholesterol concentrations (≥ 20 mol%) prevents effective β-endosulfan interaction. This behavior previously observed for lindane [17,19] and for several organophosphorus insecticides [57] suggests an inverse relationship between the partition of β-endosulfan and the molar ratio of cholesterol, as previously reported for those insecticides [15]. Conversely to β-endosulfan, α-endosulfan affects cholesterol-rich membranes (Figs. 6 and 7). Furthermore and according to the results in pure DPPC bilayers, α-endosulfan induces a new transition in membranes enriched with cholesterol at insecticide/lipid molar ratio of 1/1. Therefore, the effects of α- and β-endosulfan depend strongly on polarity, and also on cholesterol concentration.

In summary, the endosulfan isomers incorporate into lipid bilayers, perturbing the packing of lipids and affecting their thermotropic properties. Since these studies can be used to predict the localization of the xenobiotics across the thickness of the bilayer [15–21,31–33,52,57], it is tentatively suggested that the α-isomer preferentially populates the outer regions of the bilayer extending the interactions to the polar headgroups, whereas the β-isomer prefers deeper regions distributing in wider domains. Furthermore, the α-isomer promotes phase separation in lipid bilayers and, consequently, the formation of domains with high concentration of insecticide. The formation of domains may potentiate the biological activity of α-endosulfan concurring for its higher degree of toxicity, as compared with β-endosulfan. It is also worth of note that the lateral and transversal accumulation of insecticide molecules and the influence of natural membrane components, e.g. cholesterol, have to be taken into account to understand the functional interaction of the insecticides with the membranes activities.

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