*Biochimica et Biophysica Acta*, 1149 (1993) 86–92


BBAMEM 76007

# Effects of DDE on the fluidity of model and native membranes: implications for the mechanisms of toxicity

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(Received 29 December 1992)

Key words: 2,2-Bis(*p*-chlorophenyl)-1,1-dichloroethylene (DDE); Cholesterol; Fluorescent probe; Membrane fluidity

2,2-Bis(*p*-chlorophenyl)-1,1-dichloroethylene (DDE) interaction with model and native membranes was studied by means of fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), probing the bilayer core, and by intramolecular excimerization of 1,3-di(1-pyrenyl) propane (Py(3)Py), probing the outer regions of the bilayer. In the gel phase of DMPC bilayers, DDE induces concentration-dependent fluidizing effects into the hydrophobic core, but no effects are detected in the outer regions of the membrane, as evaluated by DPH and Py(3)Py, respectively. Regarding the fluid phase, DDE has no apparent effect on the bilayer center, but it induces a limited ordering effect on the outer regions. Similar effects are described for bilayers of DPPC and DSPC. Unlike DPH, Py(3)Py is very sensitive to DPPC and DSPC pretransitions, not abolished by DDE (50 $\mu$M), as opposite to the effects observed with lindane (Antunes-Madeira, M.C., Almeida, L.M. and Madeira, V.M.C. (1990) Biochim. Biophys. Acta 1022, 110–114), but similar to those observed with DDT (Antunes-Madeira, M.C., Almeida, L.M. and Madeira, V.M.C. (1991) Pestic. Sci. 33, 347–357). DDE inhibits to some extent the cholesterol-induced ordering in DMPC bilayers and high cholesterol concentrations ($\geq$ 30 mol%) do not prevent DDE interaction, as evaluated by DPH. On the other hand, the effects of DDE reported by Py(3)Py depend on temperature and cholesterol contents of DMPC bilayers. For cholesterol levels ranging from 10 to 50 mol% and temperatures below the phase transition of DMPC, Py(3)Py fails to detect any significant effect. Nevertheless, above the phase transition, Py(3)Py detects either ordering effects of DDE at low cholesterol contents ($<$ 30 mol%) or fluidizing effects at high cholesterol levels ($\geq$ 30 mol%). The results in native membranes correlate reasonably with those obtained in models of synthetic lipids. Thus, DPH does not detect any apparent effect of DDE in relatively fluid native membranes of sarcoplasmic reticulum, but detects moderate disordering effects in membranes of brain microsomes and erythrocytes, i.e., membranes with high cholesterol. On the other hand, Py(3)Py reports ordering effects of DDE in fluid membranes of sarcoplasmic reticulum, an effect similar to that observed in fluid systems of synthetic lipids without or with low cholesterol. Additionally, as described for models, Py(3)Py detects disordering effects of DDE in cholesterol rich membranes, namely, brain microsomes.

## Introduction

DDE is a stable metabolite of DDT widely distributed in the environment [3,4]. In spite of its structural similarity to DDT [3], it has little insecticidal activity in parallel with small effect on the nerve action potential [3,5]. Therefore, it has long been regarded as a detoxification product [3]. However, the effects of the compound may involve other aspects not readily comparable with the insecticide power. It has been described that DDE may exert sub lethal effects upon birds, inducing eggshell thinning and, consequently, the decline of species, especially predators and fish-eating birds. Therefore, in this respect DDE may be more active than DDT [3,4,6,7]. Furthermore, several studies indicated that DDE is as powerful as DDT to young pheasants and even more toxic than DDT to the pigeon [3]. Furthermore, the ability of DDE to inhibit ATPase enzymes such as trout brain $Mg^{2+}$-ATPase [8] and rat brain ($Na^+ + K^+$)-ATPase [9] is clearly established. DDE also inhibits the ATP-dependent $Ca^{2+}$ binding in homogenates of eggshell gland mucosa cells and decreases its $Ca^{2+}/Mg^{2+}$-activated ATPase [10,11]. The $Ca^{2+}$-ATPase of brush-border membranes from human placenta is also inhibited by DDE [12]. Additionally, DDE induces thermotropic changes [13], fluorescence polarization changes [14] and $^1$H-NMR spectral changes [15] in phospholipid dispersions. More-

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; Py(3)Py, 1,3-di(1-pyrenyl)propane; DDE, 2,2-bis(p-chlorophenyl)-1,1-dichloroethylene; DDT, 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine.

over, DDE affects the respiratory transport chain of mitochondria [16–18] and oxidative phosphorylation at the level of the ATP synthetase coupling factor (unpublished data). The effects of DDE in these biochemical and biophysical mechanisms are virtually similar to those exerted by DDT. Recent data on DDT allowed us to postulate that its toxicity may be partially modulated by fluidity changes of the membrane lipids at sensitive target sites [2,19]. Therefore, the similar structure and partition coefficients of DDT and DDE [3,20,21] suggest that biomembranes are also important target sites of DDE action. Therefore, the effects of DDE on the fluidity of well-defined model and native membranes are here under study. The term fluidity is used here in an operational sense defining it as being directly proportional to the rate of Py(3)Py intramolecular excimer formation and inversely proportional to DPH polarization. Fluidity also depends on the excited lifetimes of the probes. This fluidity is related but not identical with the physical definition of fluidity.

## Materials and Methods

*Preparation of liposomes and native membranes.* Liposomes from synthetic lipids were prepared by established procedures [22], except that buffer contained 50 mM KCl and 10 mM Tris-maleate (pH 7). Several native membranes, namely, erythrocytes, brain microsomes and sarcoplasmic reticulum were prepared as described elsewhere [23]. The protein contents were determined by the biuret method [24] calibrated with serum albumin.

In all cases, the final concentration of lipids used was nominally 345 $\mu$M. Model and native membranes were briefly sonicated to disperse aggregates and get homogeneous suspensions with a turbidity equivalent to 0.2 A measured in a Spectronic 21 spectrophotometer at 600 nm, 1 cm light path.

*Incorporation of probes and DDE into membranes.* DPH in tetrahydrofuran and Py(3)Py in ethanol were injected into membrane suspensions (345 $\mu$M in lipid) to produce lipid/probe ratios of 200 and 900, respectively. The mixtures were incubated in the dark as previously described [25,26]. Then, DDE was added from concentrated ethanolic solution (50 mM). The period of equilibration with DDE varied from 1 to 2 h, according to the concentration used. Control samples received equivalent volumes of tetrahydrofuran and ethanol. Added solvent volumes always very small (few $\mu$l) had negligible effects in the measurements.

*Fluorescence measurements.* Fluorescence spectra were recorded in a Perkin-Elmer LS 50 Spectrometer computer controlled, provided with a thermostated cell holder. The temperature of the samples was checked to an accuracy of $\pm 0.1$°C with a thermistor thermometer.

For studies with DPH, the excitation was set at 336 nm and the emission observed at 450 nm (slits of 3 nm). Fluorescence polarization ($P$) was measured as previously described [25,27–29]. A high degree of polarization reflects a limited rotational diffusion of DPH and, therefore, represents a high structural order or low membrane fluidity, and vice-versa. It should be emphasized that this fluidity is related but not identical with physic fluidity.

In studies with Py(3)Py, the excitation wavelength was at 330 nm and the excitation and emission slits of 3 and 3.5 nm, respectively. The intramolecular excimerization rate was evaluated as the excimer to monomer fluorescence intensity ratio obtained from the 490 nm to 378 nm signal ratio ($I'/I$), as previously described by Almeida et al. [26]. The excimerization is a function of rotational diffusion of the molecule about the propane bonds bridging the two pyrene-ring assemblies, increasing with membrane structural disorder. This probe is located well inside the membrane but displaced towards domains closer to the polar groups. Therefore, it preferentially distributes in the outer cooperativity regions of the bilayer [30]. Conversely, DPH preferentially distributes in the fluid bilayer core [27,31,32].

All the fluorescence measurements were corrected for the contribution of light scattering by using controls with membranes, but without added probes. Control experiments with increasing concentrations of DDE in solvents (alcohol and liquid paraffin), from 0 to 70°C, have shown that no significant fluorescence quenching, upon the steady state fluorescence polarization of DPH and the fluorescence intensity ratio $I'/I$ of Py(3)Py, is exerted by DDE at the concentrations used in this study.

*Reagents.* Cholesterol, dimyristoyl-, dipalmitoyl- and distearoylphosphatidylcholines and DPH were obtained from Sigma. The probe Py(3)Py was a gift of Dr. Zachariasse from Max-Planck-Institut für Biophysikalische Chemie (Göttingen, Germany). DDE (chromatographic grade) was obtained from Chemservice, (West Chester, UK).

## Results and Discussion

*Thermotropic profile of DMPC*

The effects of DDE (0–100 $\mu$M) on the fluorescence polarization of DPH and on the fluorescence intensity ratio ($I'/I$) of Py(3)Py, embedded into bilayers of DMPC are summarized in Fig. 1. The fluorescence polarization of DPH significantly decreases with increasing concentrations of DDE in the gel phase of DMPC (temperature below the transition) and is not altered in the fluid phase (Fig. 1A). Additionally, broadening of the cooperative phase transition is observed upon DDE interaction, suggesting that the size

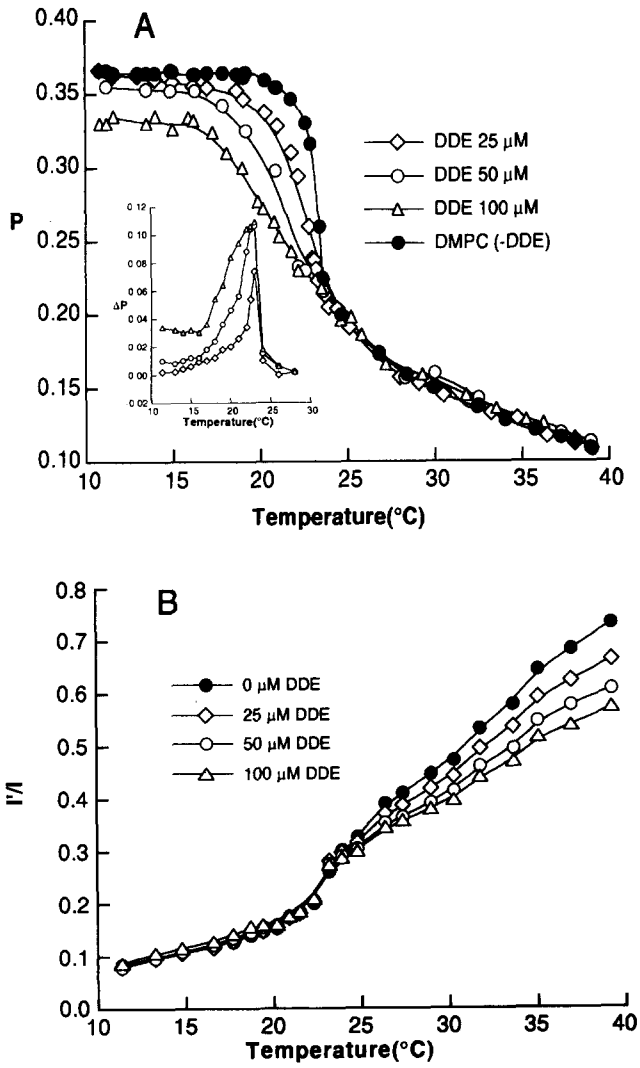


Fig. 1. Effects of DDE on the thermotropic phase transition of DMPC determined by fluorescence polarization of DPH (A) and by Py(3)Py excimerization, $I'/I$ (B). The inset represents differential polarization of DPH ($\Delta P$) as a function of temperature, at several DDE concentrations. Maximal effect of DDE is close to the main transition temperature.

of the cooperative unit is reduced (Fig. 1, inset). The strongest effect of DDE is observed at temperatures approaching that of the cooperative phase transition (Fig. 1A, inset). Probably, at this temperature the incorporation of the insecticide is favored as described for other insecticides, namely, DDT, lindane, malathion and parathion [23]. However, a strong incorporation is not the only explanation for the effect on fluidity, since significant incorporation occurs in the fluid phase [20] without a corresponding effect on DPH polarization. These results with DPH are consistent with those obtained by Buff et al. in DPPC bilayers [14]. They also found dose-related effects of DDE and of other insecticides only in gel phase. On the other hand, the excimer/monomer fluorescence intensity ratio ($I'/I$) of Py(3)Py is only perturbed by DDE in the fluid phase of DMPC (Fig. 1B). The differential effects of DDE below and above the phase transition of DMPC, as detected by DPH and Py(3)Py, are probably related to the larger fluidity gradient across the thickness of the bilayer in the gel as compared with the fluid phase [33–35]. Consequently, the distribution of DDE and its effects are modulated by the bilayer fluidity gradient. The fluid interior of the bilayer, in the gel condition, induces a preferential local distribution of DDE with a consequent increase in fluidity, reported by DPH also located in the bilayer core [27,31,32]. Py(3)Py excimerization is silent to this modification owing the probe location in the outer regions of the bilayer [26], not reached by DDE in the gel phase of DMPC. In the fluid phase of DMPC, DPH detects no apparent effect of DDE and Py(3)Py reports ordering effects. Above the phase transition, the entire bilayer remains fluid, although a fluidity gradient is still apparent, with the inner core more fluid relatively to domains closer to the surface [35]. DDE will putatively extend non-selectively at any depth with a wider distribution across the thickness of the bilayer as compared with a narrower distribution in the gel phase. Therefore, DPH, located in the fluid bilayer core, detects no apparent effect of DDE but Py(3)Py, in the outer regions, reports ordering effects of the compound, inducing here an increase in packing density. Therefore, in the fluid phase, the outer regions of the bilayer are ordered by DDE with no detectable effect in the bilayer center. Some of the described events could also arise from DDE effects upon the lifetimes of the probes. However, these effects are probably negligible owing to the absence of DDE effects in the higher temperature range for DPH and the lower temperature range for Py(3)Py (Figs. 1 and 2).

In conclusion, the results summarized in Fig. 1 indicate that DDE essentially fluidizes the hydrophobic core of the bilayer, in the gel phase, but in the fluid phase it exerts condensing effects in the outer regions.

*Comparative effects on DMPC, DPPC and DSPC thermograms*

The effects of DDE on the thermograms of DPPC and DSPC bilayers detected by DPH and Py(3)Py are essentially similar to those described for DMPC (Fig. 2). However, the shifting and broadening induced by DDE and detected by DPH, are more pronounced in DMPC as compared with DPPC and DSPC (Fig. 2A). Thus, for 50 $\mu$M DDE, Tm of DMPC is shifted by about 2 C° and $T_m$ of DPPC and DSPC are shifted by about 1 C°. These values are intermediate between those obtained for lindane [36] and DDT [19], suggesting also intermediate perturbations of phospholipid intermolecular interactions.

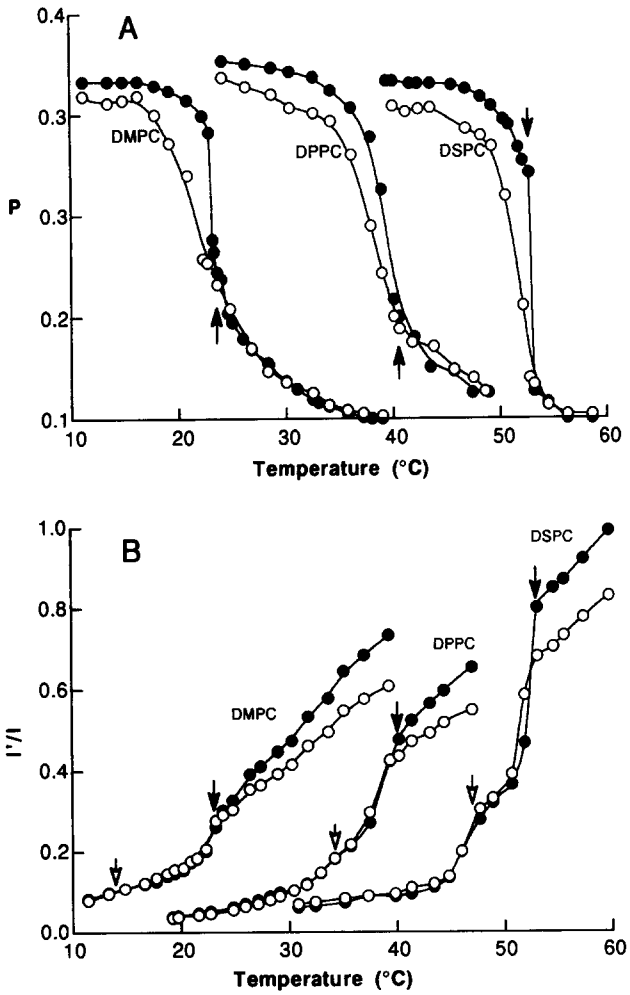


Fig. 2. Thermotropic phase transition profiles of DMPC, DPPC and DSPC bilayers evaluated by fluorescence polarization of DPH (A) and intramolecular excimerization of Py(3)Py (B), in the absence (solid symbols) and presence (open symbols) of 50 $\mu$M DDE. Unlike DPH, Py(3)Py is very sensitive to pretransitions not removed by DDE. The arrows indicate the position of main transitions (solid arrowheads) and pretransitions (open arrowheads).

As described in Fig. 2B, the ordering effects promoted by DDE in outer regions of the bilayer, in the fluid phase, are more pronounced in DSPC as compared with the effects in the other lipid species. Data of Fig. 2B also indicate that the pretransitions of DPPC and DSPC, detected by Py(3)Py, are not removed by 50 $\mu$M DDE (Fig. 2B), an effect similar to that described, previously, for DDT [2]. This finding further suggests a preferential localization of DDE into the fluid bilayer center without directly affecting the cooperativity region where the events determining pretransitions occur. Apparently, this region is only reached by the insecticide in the fluid phase. A preferential distribution in the bilayer center of gel phase has been previously postulated for DDT [2]. Interesting is also the fact that lindane, unlike DDE and DDT [2], removes the pretransitions [1] an effect identical to that of cholesterol [37], suggesting that lindane aligns with the aliphatic chains of membrane lipids in the cooperativity region. Therefore, insecticide structure is a main parameter controlling its localization along the thickness of the bilayer, and, certainly, the specific membrane effects pertaining to the toxicology behavior of the various compounds.

*Thermotropic behavior of DMPC bilayers enriched with cholesterol*

The results detected by DPH and Py(3)Py in DMPC bilayers enriched with cholesterol are represented in Figs. 3 and 4. Cholesterol progressively increases the molecular order in fluid DMPC bilayers and high levels of cholesterol endow the bilayer with a physical state intermediary between the gel and the liquid-crystalline state. Consequently, the cooperative phase transition vanishes [38]. The effects of DDE in DMPC bilayers with low cholesterol ($< 30$ mol%) are similar to those described above for pure DMPC. Thus, DDE essentially fluidizes the hydrophobic core of the bilayer in the gel phase as detected by DPH (Fig. 3) and condenses the outer regions, in the fluid phase, as reported by Py(3)Py (Fig. 4), suggesting the separation of DMPC-DDE rich domains from those containing cholesterol. Furthermore, DMPC membranes enriched with high cholesterol contents ($\geq 30$ mol%) are fluidized by DDE in the interior and in the outer regions of the bilayer as revealed by DPH and Py(3)Py, respectively. These effects reflect an increase in the intermolecular distances of the phospholipids in order to accommodate cholesterol and DDE. A detailed examination of Fig. 4A,B indicates that below the transition of DMPC, Py(3)Py fails to detect any effect of DDE in cholesterol-poor and rich membranes, since the insecticide accumulates in the highly fluid hydrophobic core of the bilayer [39] where the fluidity is further increased by its presence as probed by DPH. The disordering effects towards the upper regions of the bilayer promoted by the insecticide and also by the increase in temperature induce diffusion of DDE to the upper regions where cholesterol concentration dictates either order or disorder (Figs. 4A and B).

In conclusion, data in model membranes clearly indicate that DDE populates the fluid domains of the bilayer, i.e., the inner core in the gel phase and the entire width in the fluid phase.

*Native membranes*

Sarcoplasmic reticulum (SR), brain microsomes (BM) and erythrocytes (E), differing in intrinsic cholesterol content, were chosen to study the effects of DDE in the temperature range from 10 to 40°C (Figs. 5 and 6). Although in this range the native membranes remain in the fluid state [40], the relative degree of

fluidity changes with temperature and intrinsic cholesterol (Figs. 5 and 6, solid lines). Thus, membranes of sarcoplasmic reticulum with low cholesterol content (approx. 6 mol%) are significantly more fluid than those of brain microsomes and erythrocytes, where cholesterol accounts for 25 and 37 mol%, respectively, as reported by the probes DPH and Py(3)Py.

The effects of DDE in native membranes (dotted lines) probed by DPH polarization (Fig. 5A,B) are similar to those described for models. Thus, DDE does not exert apparent disordering effects into relatively fluid native membranes of sarcoplasmic reticulum but moderate disordering effects of DDE are detected by DPH in cholesterol-rich membranes, namely, brain microsomes and erythrocytes. Conversely to diphenylhexatriene data, bispyrenylpropane reports ordering effects of DDE in fluid membranes of SR, similarly to the effects described in fluid synthetic lipids without or with low cholesterol contents (Fig. 6A,B). Again, as in the models of synthetic lipids disordering effects of

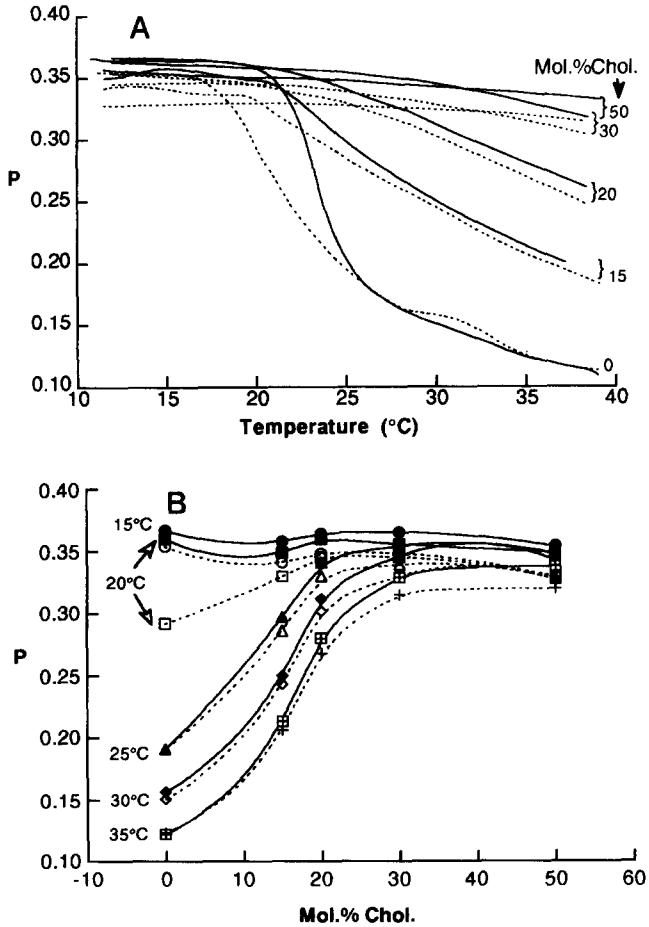


Fig. 3. Fluorescence polarization of DPH in DMPC bilayers enriched with cholesterol as a function of temperature (A) and intrinsic cholesterol content (B), in absence (solid lines and symbols) and presence (dotted lines and open symbols) of 50 $\mu$M DDE. In A, each curve has been drawn across 17–20 experimental points which were removed for the sake of clarity.

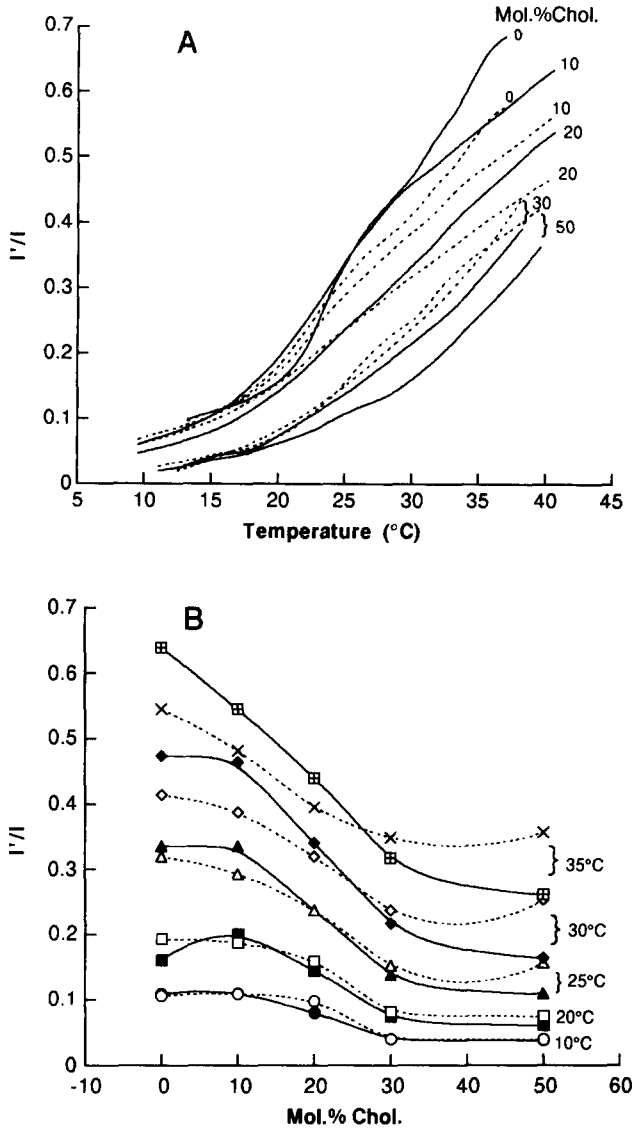


Fig. 4. Py(3)Py intramolecular excimerization in DMPC bilayers enriched with cholesterol as a function of temperature (A) and cholesterol content (B), in absence (solid lines and symbols) and presence (dotted lines and open symbols) of 50 $\mu$M DDE. In A, each curve has been drawn as described for Fig. 3.

DDE are detected by Py(3)Py in cholesterol-rich membranes, such as brain microsomes (Fig. 6A,B) and erythrocytes (results not shown here). The ordering and disordering effects promoted by DDE in the upper regions of the bilayer are better noticed at high temperatures, indicating that these regions are more accessible to DDE. Thus, the temperature and cholesterol concentration are main parameters controlling DDE effects. Furthermore, there is a critical cholesterol concentration below and above which DDE has either ordering or disordering effects, respectively (Fig. 6B). Apparently, with the increase in cholesterol contents, the geometry of the bilayer changes and the diffusion of the insecticide to the upper regions induces an increase in phospholipid intermolecular contacts to

accommodate both cholesterol and DDE. Consequently, disordering effects are detected by Py(3)Py. If cholesterol concentration is low, DDE diffuses into cholesterol poor domains and orders these regions due to an increase in packing density.

Described findings are similar to those obtained for DDT [2,19], except that in cholesterol-rich membranes, namely brain microsomes, Py(3)Py detects disordering DDE effects (present study) and ordering DDT effects. Therefore, DDT will probably exert a stronger influence on regions free or poor in cholesterol, e.g., most lipid–protein interfaces [41]. Since nerve membranes have high cholesterol contents [42], a causal relationship between the acute insecticidal action of DDT and its preferential effects in lipid domains surrounding the $Na^+$-channel may be predicted.

The effects of DDE on membrane fluidity certainly contribute for its effects in membrane linked functions [4,7–12,17,18,43,44] and, consequently, for its toxicology behavior.

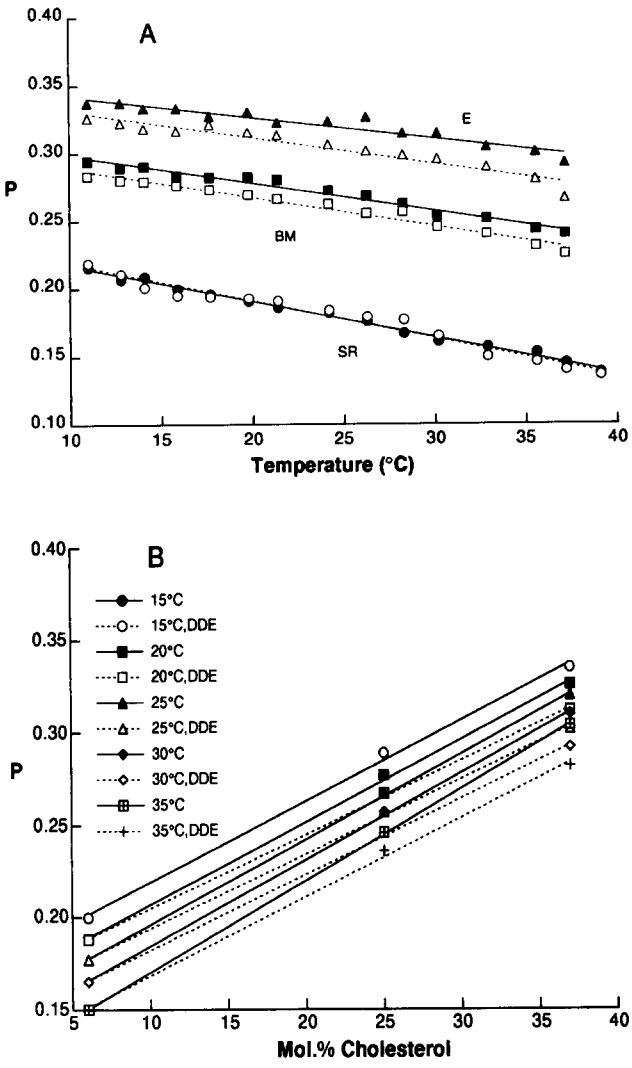


Fig. 5. Fluorescence polarization of DPH in native membranes as a function of temperature (A) and intrinsic cholesterol content (B), in absence (solid symbols) and presence (open symbols) of 50 $\mu$M DDE. Fluorescence polarization data of (B) were taken from (A) at 15, 20, 25, 30 and 35°C. Cholesterol/phospholipid molar ratios for sarcoplasmic reticulum (SR), brain microsomes (BM) and erythrocytes (E) are 6, 25 and 37 mol%, respectively.

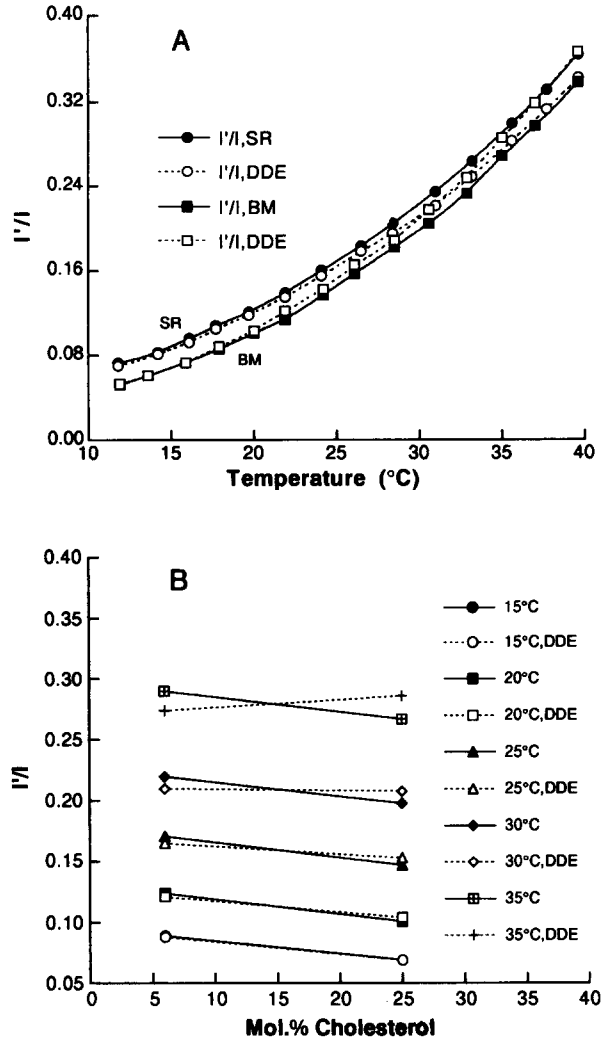


Fig. 6. Intramolecular excimerization of Py(3)Py, in sarcoplasmic reticulum and brain microsomes as a function of temperature (A) and intrinsic cholesterol content (B) in absence (solid symbols) and presence (open symbols) of 50 $\mu$M DDE. Cholesterol/phospholipid molar ratios for native membranes is indicated in Fig. 5. Error bars are not represented, since for most experimental points they are encompassed by the size of the symbols.

## Acknowledgement

This work was supported by grants from INIC and JNICT.

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