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# Effects of lindane on membrane fluidity: intramolecular excimerization of a pyrene derivative and polarization of diphenylhexatriene

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**Fluorescence polarization studies of 1,6-diphenyl-1,3,5-hexatriene (DPH) have been compared with the excimer/monomer fluorescence intensity ratio ($I'/I$) of 1,3-di(2-pyrenyl)propane, (2Py(3)2Py). This ratio permits evaluation of changes in fluidity of the outer regions of the bilayer, where 2Py(3)2Py preferentially distributes. On the other hand, fluorescence polarization of DPH reports the structural order of the bilayer core. In the fluid phase of DMPC bilayers, for lindane concentrations higher than 25 $\mu$M, the excimer/monomer fluorescence intensity ratio ($I'/I$) decreases, thus reflecting an order increase of the probe environment. However, in the same conditions, the fluorescence polarization of DPH is almost insensitive to any perturbation. Identical results have been obtained in other pure lipid bilayers, namely DPPC and DSPC. However, both probes detect disordering effects of lindane in the gel phase of these lipids. The pyrene probe, unlike DPH, is very sensitive to the pretransitions of DPPC and DSPC, removed in the presence of lindane. Both probes fail to detect any apparent effect of lindane in DMPC bilayers enriched with high cholesterol content (> 30 mol%). However, in DMPC bilayers with low cholesterol content (< 30 mol%), for temperatures below the phase transition of DMPC, both probes detect fluidizing effects induced by lindane. Nevertheless, above the phase transition of DMPC, 2Py(3)2Py detects ordering effects of lindane, whereas DPH detects hardly any effect. These results in DMPC bilayers with low cholesterol content are qualitatively similar to those described for DMPC without cholesterol.**

## Introduction

Studies in our and other laboratories [1–7] suggest that most of the physiological effects of lindane, either acute or chronic, appear to be membrane-connected. In attempt to understand and rationalize lindane–membrane interactions, the accumulation of the compound into model and native membranes has been studied [8–11]. It was concluded that membrane fluidity is the main parameter affecting lindane incorporation and, probably, its toxicity. Furthermore, the incorporation itself affects the general order of the membrane as evaluated from the fluorescence polarization of 1,6-diphenylhexatriene (DPH), a probe located in the bilayer interior [12–14]. The above results and the postulated gradient of fluidity across the width of the bilayer [15] indicate that lindane distribution as a function of depth in the bilayer may need to be considered. Thus, the present work compares the fluorescence polarization of DPH with the excimer/monomer fluorescence intensity ratio, $I'/I$, of 2Py(3)2Py, a probe sitting closer to the polar region. Indeed, polarity and polarizability studies with a very similar probe (1Py(3)1Py) and other pyrene analogues are consistent with a membrane location, not in the very hydrophobic core, but somewhere closer to the polar headgroups of the phospholipids [16,17]. The probe used in the present studies is very similar to 1Py(3)1Py, which has been extensively used in our laboratory as a fluidity probe [16,18,19]. Despite the usefulness of 1Py(3)1Py [16–21], recent studies indicate that one monomer and two excimer species are in-

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; lindane, γ-1,2,3,4,5,6-hexachlorocyclohexane; 1Py(3)1Py, 1,3-di(1-pyrenyl)propane; 2Py(3)2Py, 1,3-di(2-pyrenyl)propane.

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volved, whereas 2Py(3)2Py shows simpler kinetics with only one excimer [22]. In this work, we have used 2Py(3)2Py instead of 1Py(3)1Py.

## Materials and Methods

*Preparation of liposomes*

Solutions of pure phospholipids in $CHCl_3$ were taken in round bottom flasks and the solvent was evaporated to dryness. The resulting lipid film was then hydrated with an appropriate volume of buffer (50 mM KCl/10 mM Tris-maleate (pH 7.0)) and dispersed under a $N_2$ atmosphere by handshaking in a water bath, 7–10 °C above the transition temperature of the phospholipids. This liposome suspension was then mixed vigorously by vortexing and briefly sonicated to disperse large lipid aggregates. Phospholipid-cholesterol bilayers were obtained by supplementing original phospholipid solutions with appropriate amounts of cholesterol. The final concentration of lipid was nominally 345 $\mu$M in all cases.

*Incorporation of the probes and lindane into membranes*

DPH (2 mM) in tetrahydrofuran was injected, while vortexing, into membrane suspensions (345 $\mu$M lipid) to give a final phospholipid/probe molar ratio of about 200. The mixture was then incubated, in the dark, for 18–20 h.

The incorporation of 2Py(3)2Py into membranes was carried out as described previously [18]. Aliquots of an ethanolic solution of the probe ($4 \cdot 10^{-5}$M), were added to the membrane suspension (345 $\mu$M lipid) to give a final probe concentration of about $4 \cdot 10^{-7}$M, i.e., a phospholipid/probe molar ratio of about 900. The mixture was initially vigorously vortexed for 10 s, at a temperature 10 °C above the main transition temperature of the lipid and then incubated overnight with gentle stirring.

After the period of incubation with the probes, lindane was added from concentrated ethanolic solutions (50 mM). The period of equilibration with lindane varied from 1–2 h according to the concentration used.

Control samples received equivalent volumes of tetrahydrofuran and ethanol.

*Fluorescence measurements*

Fluorescence spectra were measured in a Perkin-Elmer spectrofluorometer, Model MPF-3, 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. Excitation at 336 (rather than at 360 nm, as normally used) had the purpose to eliminate light scattering and to avoid detectable quenching effects of lindane on DPH static fluorescence signals. The excitation and emission bandwidths were 4 and 6 nm, respectively. The degree of fluorescence polarization ($P$) was calculated, according to Shinitzky and Barenholz [23] and Shinitzky and Inbar [24], 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 emitted light whose plane of polarization is oriented, respectively, parallel and perpendicular to the plane of polarization of the excitation beam. $G$ is the correction factor for instrument polarization, given by the ratio of vertically to the horizontally polarized emission components when the excitation light is polarized in the horizontal direction [25]. A high degree of fluorescence polarization ($P$) represents high structural order and/or low membrane fluidity, and vice-versa. In studies with 2Py(3)2Py, the excitation wavelength was 338 nm and the excitation and emission bandwidths were 5 nm. The intramolecular excimerization rate was evaluated as the excimer to monomer fluorescence intensity ratio obtained from the 480 nm to 376 nm signal ratio ($I'/I$), in a similar way to that described previously for 1Py(3)1Py [18]. These measurements as well those with DPH were always corrected for the contribution due to light scattering by using controls with membranes but without added probes. The excimer to monomer fluorescence intensity ratio, $I'/I$, for intramolecular pyrene excimer probes, changes with the fluidity of viscous media and has been used to monitor the fluidity of membranes [16–21]. The excimer/monomer fluoresence intensity ratio, $I'/I$, increases with membrane fluidity and decreases with membrane order.

Control experiments with increasing concentrations of lindane either in lipid bilayers or in several solvents (alcohols and liquid paraffin) from 0 to 70 °C have shown that no fluorescence quenching upon the static fluorescence signals is exerted by lindane at the concentrations used in this study. However, this does not completely exclude effects of lindane on fluorescence lifetimes not measured in this work, since appropriate facilities are lacking in our laboratory. Since only static signals were measured, we are confident that lindane does not induce artifacts in the measurements.

*Reagents*

Crystalline DPH was obtained from Sigma Chemical Co. The probe 2Py(3)2Py synthesized as described in Ref. 22 was a gift of Dr. Zachariasse. Lindane (chromatography standard) was purchased from Supelco. All the other chemicals were of research grade.

## Results and Discussion

*Effects of lindane in pure phospholipid bilayers*

The main purpose of this study was to compare the relative perturbations induced by lindane in different regions across the bilayer thickness. Thus, 2Py(3)2Py was chosen to probe the fluidity of the outer regions, whereas DPH was used to report the structural order of the bilayer core. These two probes were selected since they report similar physical parameters and their locations across the membrane have been well characterized [12–14,16–18]. Thus, DPH polarization reports the rotational diffusion of the probe and strongly depends on fluidity [23–25]. On the other hand, the intramolecular excimerization of 2Py(3)2Py is determined by the rate of motion about the $\sigma$ bonds linking the two pyrene rings and depends on the fluidity of probe environment [16–18].

The effects of increasing concentrations of lindane on the fluorescence polarization of DPH and on the fluorescence intensity ratio ($I'/I$) of 2Py(3)2Py, embedded into bilayers of DMPC is shown in Fig. 1. The fluorescence polarization of DPH significantly decreases with increasing concentrations of lindane in the range of temperature from about 17°C to the main transition temperature centered at 24°C, as expected from the partition coefficients of lindane [10]. The strongest effect is noticed at about 21°C, i.e., at a temperature approaching that of the cooperative phase transition, which favours the incorporation of lindane in DMPC bilayers [10]. However, this favoured incorporation of lindane is not the only explanation for its effect on fluidity, since significant incorporation occurs at 10 and 30°C without a corresponding effect on DPH polarization. Therefore, it appears that lindane increases the tendency for phase separation of fluid and gel domains by promoting dislocations in the bilayer lipid lattice as the temperature approaches the critical point of the phase transition. Clearly, lindane broadens the transition profile, thus, decreasing the size of the cooperative unit and shifts the midpoint to lower temperature values (e.g., 20°C for 100 $\mu$M lindane). The shift and broadening of the phase transition midpoint of DMPC bilayers are also detected by 2Py(3)2Py (Fig. 1B). Furthermore, the shift and broadening of the transition event detected by both probes depend on the insecticide concentration up to about 100 $\mu$M.

According to Jain and Wu [26], a shifting and broadening of the transition profile would mean a localization of the foreign molecules in the vicinity of the first eight carbons of the acyl chains, i.e., in the cooperativity region. Such a localization is similar to that of the rigid rings of cholesterol, i.e., the bilayer region most perturbed by this compound [27,28]. Thus, a localization of lindane indentical to that of cholesterol is here tentatively suggested.

Furthermore, for lindane concentrations higher than 25 $\mu$M and for temperatures above the phase transition of DMPC, the excimer/monomer fluorescence intensity ratio ($I'/I$) decreases, reflecting a decrease in fluidity of probe environment. Conversely, lindane concentrations ranging from 10 to 100 $\mu$M have almost no effect on the fluorescence polarization of DPH. These results with DPH are consistent with those obtained by Jones and Lee [11] in dioleoylphosphatidylcholine bilayers. Additionally, DDT and other insecticides also have no effect on the fluorescence polarization of DPH embedded in fluid bilayers of DPPC [29]. The differential

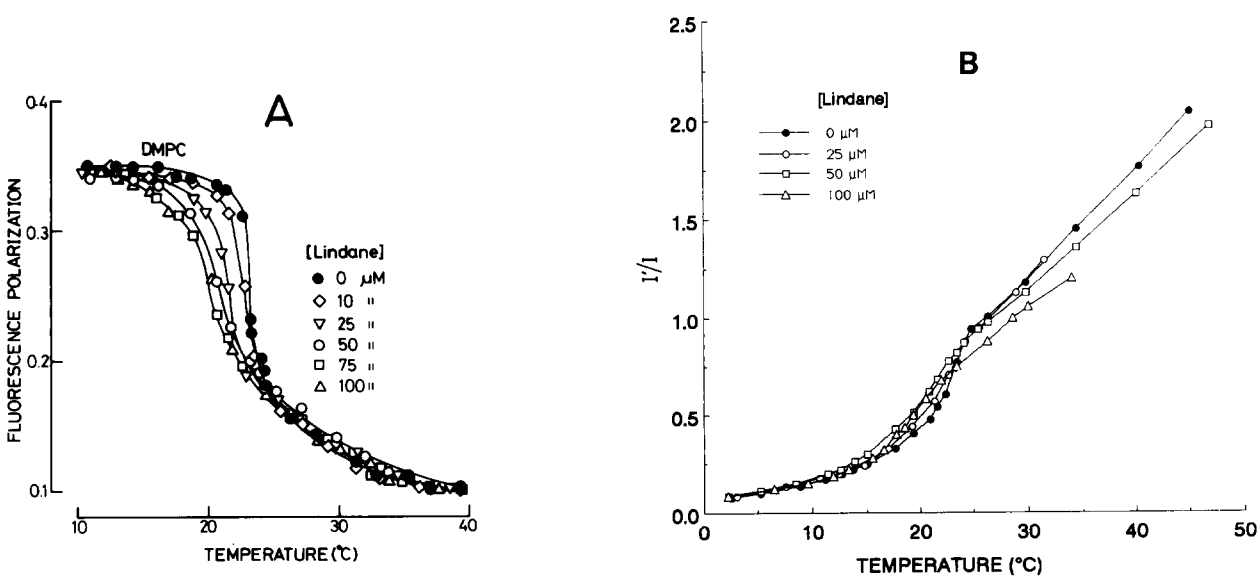


Fig. 1. Effects of lindane on the thermotropic phase transition of DMPC determined by fluorescence polarization of DPH (part A) and by intramolecular excimerization of 2Py(3)2Py, i.e., monomer/excimer ratio $I'/I$ (part B).

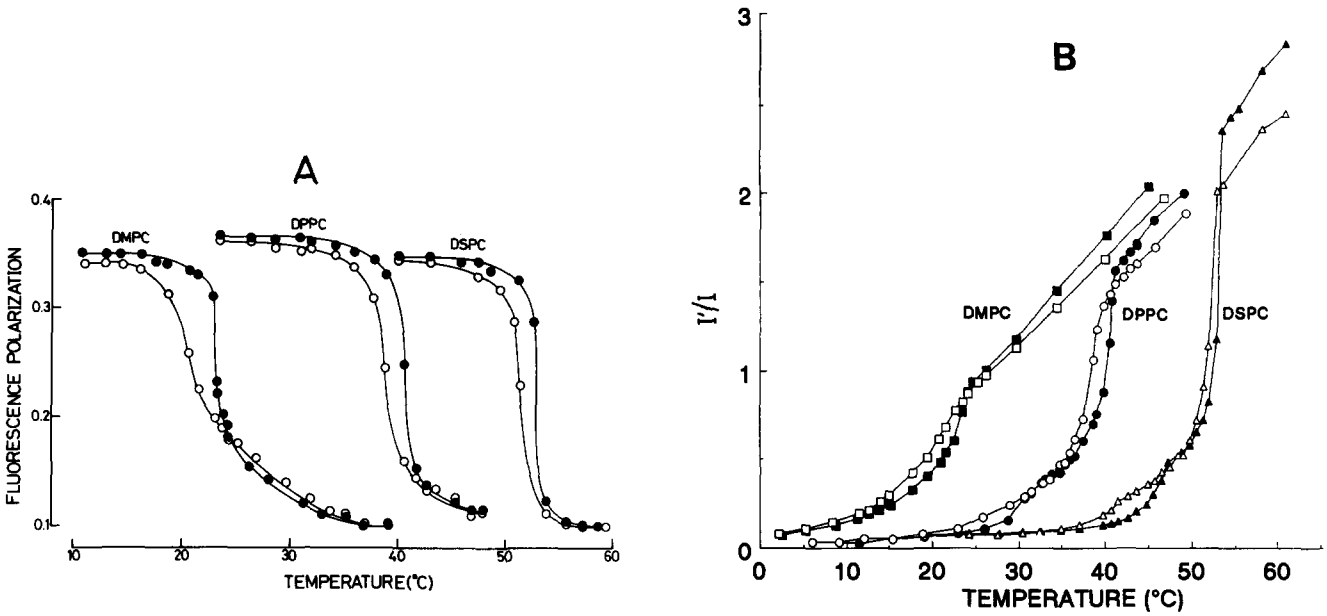


Fig. 2. Thermotropic phase transitions of DMPC, DPPC and DSPC bilayers determined, in the absence (solid symbols) and in the presence (open symbols) of 50 $\mu$M lindane, by fluorescence polarization of DPH (A) and intramolecular excimerization of 2Py(3)2Py (B).

effects of lindane in the fluid phase of DMPC bilayers, detected by the above probes, DPH and 2Py(3)2Py, is interpreted on the basis of the insecticide localization along the thickness of the bilayer and the fluidity gradient of the bilayer. Let us assume that, in the fluid phase, the fluidity of the inner bilayer core is large as compared to the upper regions of the bilayer [15] and that lindane is localized in the cooperativity region. Consequently, the insecticide would affect the fluidity of the interior proportionately less than that of the external regions of the bilayer. Additionally, no disordering is induced in these regions due to an increase in packing density. Therefore, it is not very surprising that 2Py(3)2 Py detects order and DPH detects no apparent effect of lindane in fluid bilayers of DMPC.

Data of Fig. 2 indicate that pretransitions of DPPC and DSPC bilayers are clearly detected by 2Py(3)2Py. The relative insensitivity of DPH to the pretransitions

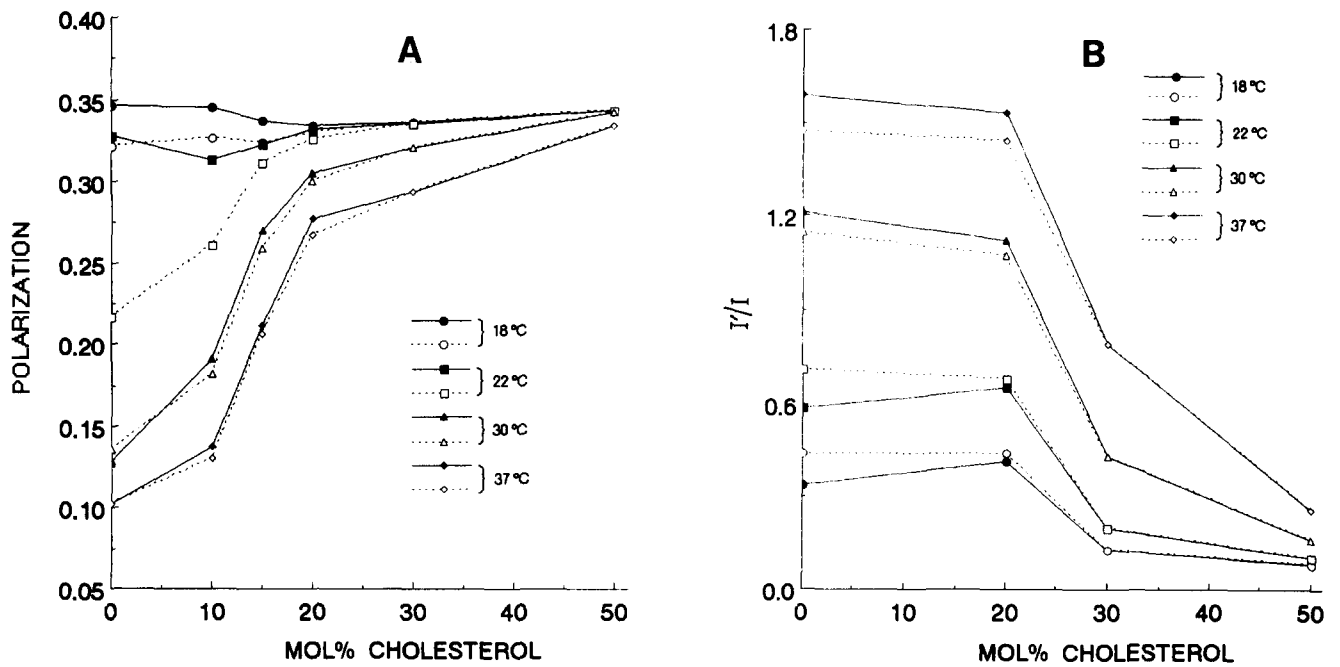


Fig. 3. Fluorescence polarization of DPH (A) and intramolecular excimerization of 2Py(3)2Py (B) in DMPC bilayers enriched with increasing concentrations of cholesterol (0–50 mol%), in the absence (solid symbols) or in the presence (open symbols) of 50 $\mu$M lindane.

of phosphatidylcholines is to be expected, considering the localization of the probe in the inner core of the bilayer [13,14]. The detection of pretransitions by 2Py(3)2Py further supports the conclusion that the probe preferentially distributes in the upper regions of the bilayer. Lindane (50 $\mu$M) removes DPPC and DSPC pretransitions, an effect similar to that of cholesterol [30], suggesting that lindane aligns with the aliphatic chains of membrane lipids in the cooperativity region.

The overall effects of lindane (50 $\mu$M) on the thermotropic behaviour of DPPC and DSPC bilayers detected by DPH and 2Py(3)2Py are similar to those described for DMPC (Fig. 2). However, the fluidizing effects of lindane are higher in DMPC than in DPPC and DSPC. Since DMPC lipid species are intrinsically more fluid than DPPC and DSPC [31], it appears that the fluidizing effects of lindane are modulated by the fluidity itself. On the other hand, the ordering effects of lindane detected by 2Py(3)2Py above the phase transition are larger in DSPC as compared with the other more fluid species (Fig. 2B). As illustrated in Fig. 2A, the fluorescence polarization of DPH is almost insensitive to any perturbation in the temperature range above the main phase transition of any lipid species.

*Effects of lindane in phospholipid-cholesterol bilayers*

Both probes detect fluidizing effects of lindane in DMPC bilayers with low cholesterol content ($< 30$ mol%) at temperatures below the phase transition of DMPC, (Fig. 3). However, above the phase transition of DMPC, 2Py(3)2Py detects ordering effects of lindane, whereas DPH detects hardly any effect of the insecticide. Consequently, it appears that lindane disorders the upper regions and the inner core of the bilayer, in the gel phase of DMPC. Nevertheless, in the fluid phase, lindane behaves differently at different depths of the bilayer, since it orders the upper region and has almost no effect in the hydrophobic inner core, similarly as described for DMPC without cholestrol.

Data in Fig. 3 also reveals that DPH and 2Py(3)2Py fail to detect any effect of lindane in DMPC bilayers enriched with high cholesterol content ($> 30$ mol%). These findings fully agree with previous partition studies showing that cholesterol effectively depresses the incorporation of lindane. Actually, the insecticide is almost completely excluded from the membrane at 50 mol% cholesterol. The sterol effectively withdraws lindane from the membrane since it occupies the domains where lindane is accommodated.

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