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Interactions of pyrethroids with phosphatidylcholine liposomal membranes

Keith J. Stelzer and Michael A. Gordon

Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas College of Health Sciences and Hospital, Kansas City, KS 66103 (U S A)

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Interactions of several pyrethroids with membrane lipids in the form of dipalmitoylphosphatidylcholine (DPPC) liposomes have been studied using fluorescent membrane probes. Fluorescence anisotropy values and lifetimes (determined by phase-shift and demodulation techniques) of the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene, were decreased in gel phase liposomes by pyrethroids at concentrations on the order of 10 μM. The pyrethroids containing a cyano substituent were also observed to cause collisional quenching of diphenylhexatriene fluorescence. Pyrethroids differed in their effectiveness at lowering the phase transition temperature of DPPC, and in their ability to broaden the temperature range of this transition. The fluorescence intensity of DPPC-incorporated chlorophyll *a* was used to monitor the pretransition of DPPC and the lateral diffusion of a membrane component located in the polar headgroup region. Permethrin did not affect chlorophyll *a* fluorescence intensity at any temperature. It may be concluded from these results that pyrethroids are preferentially located in the interior hydrophobic regions of the lipid bilayer, and that these compounds can disorder hydrocarbon packing in the bilayer core. However, polar headgroups were not disordered, and diffusion of membrane components in the polar headgroup region was not altered.

Introduction

Model membrane systems composed of phospholipids have been used extensively to study the interactions of a variety of drugs [1–15] and toxicologic agents [16–25] with membrane lipids. It has recently been reported that pyrethroids disrupt membrane lipid packing order in murine splenic lymphocytes [26]. To characterize further the interactions between pyrethroids and membrane lipids, we have studied these compounds in a model membrane system consisting of large multilamellar DPPC liposomes.

Pyrethroids are synthetic esters which may vary in structure at both the alcohol and acid moieties. This class of compounds is derived from the naturally occurring substance, pyrethrum. Pyrethroids are generally categorized according to the structure of the alcohol moiety. The earliest of the pyrethroids to be synthesized were similar to pyrethrum in that they contained an alkenylmethylcyclopentenolone alcohol [27]. Photostability of pyrethroids was later enhanced with the utilization of a phenoxybenzyl alcohol moiety [27]. The most recently developed pyrethroids contain a cyano substituent at the α-carbon of the phenoxybenzyl alcohol [27].

Many of the cellular effects of pyrethroids involve membrane-related structures such as sodium channels [28–39], receptor-ionophore complexes [40–43], and ATPases [44–46]. In addition, cell membrane phospholipid composition may be a determining factor in the resistance of strains of *Musca domestica* to the neurobiological effects of pyrethroids [47]. The binding of pyrethroids, stereospecifically, to active sites is probably of major

importance to pyrethroid neural activity [42,43]. However, at the lowest pyrethroid concentration necessary for specific binding site saturation in mouse brain tissue (0.1 μM), 95% of total tissue binding was non-specific and unsaturable [48]. It is evident that an understanding of pyrethroid-membrane interactions is required in order to define fully how and where these compounds may affect cellular function.

In the current study we have examined the concentration- and temperature-dependent effects of pyrethroids on model membrane lipid packing order by utilizing the fluorescent membrane probes, diphenylhexatriene and chlorophyll *a*. The pyrethroids included in this study may be separated into three types based upon the alcohol moiety: nonphenoxybenzyl (allethrin), phenoxybenzyl (permethrin), and cyano (cypermethrin, fluvalinate, fenpropathrin).

Materials and Methods

Chemicals. DPPC, chlorophyll *a*, and 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene (dimethyl POPOP) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Diphenylhexatriene was purchased from Molecular Probes, Inc., Junction City, OR, U.S.A. The following pyrethroids were gifts from the companies indicated: permethrin and cypermethrin (FMC Corp., Princeton, NJ, U.S.A.), fenpropathrin (Chevron Chemical Co., Richmond, CA, U.S.A.), allethrin (McLaughlin Gormley King Co., Minneapolis, MN, U.S.A.), fluvalinate (Zoecon Corp., Palo Alto, CA, U.S.A.). All pyrethroids were of analytical grade. Other compounds used in this study were of the highest commercially available quality.

Labeling of liposomes with diphenylhexatriene. Large multilamellar liposomes were prepared from a solution of DPPC which was dissolved in chloroform. The chloroform was evaporated under nitrogen, and the resulting lipid was subjected to vacuum for 5 min. The DPPC was then suspended in 0.01 M phosphate-buffered saline (PBS, pH 7.4) to give a lipid concentration of 1 mM. Following a 15 min incubation at 51°C, the suspension was vortexed for 5 min. An equal volume of 2 μM diphenylhexatriene suspended in phosphate-buffered saline was added to the liposomal suspension, giving a lipid/probe molar ratio of 500 1. The suspension was then incubated for 45 min at 25°C. After incubation, the liposomal suspension was diluted in phosphate-buffered saline to give a final DPPC concentration of $6 \cdot 10^{-5}$ M for fluorometric measurements.

Labeling of liposomes with chlorophyll a DPPC liposomes were labeled with chlorophyll *a* using a modification of a previously described technique [49,50]. Chloroform was evaporated from DPPC as described above, with the exception that chlorophyll *a* was included in the chloroform solution such that the lipid/chlorophyll *a* molar ratio was 400 : 1. Phosphate-buffered saline was added to give a $1.6 \cdot 10^{-4}$ M DPPC suspension, and this suspension was incubated for 15 min at 51°C. Following vortexing for 5 min, the DPPC suspension was used directly for fluorescence measurements.

Fluorescence measurements Fluorescence anisotropy measurements and phase-shift and demodulation measurements, for computation of fluorescence lifetimes, were carried out with diphenylhexatriene-labeled liposomes. For anisotropy measurements, an SLM 8000 fluorometer was configured in the T-format with polarizers in the excitation and emission beams [51]. Data were transferred directly to a Hewlett-Packard 87 computer for analysis and storage. As previously described [52,53], incident light (360 nm) was polarized first horizontally then vertically An SLM MC320 monochromater set at 430 nm was placed in the vertically polarized emission beam, and a Schott KV-389 cut on filter was placed in the horizontally polarized emission beam. Pyrethroids showed no detectable absorbance of light at the excitation and emission wavelengths.

An SLM 4800 fluorometer, configured as previously described [53], was utilized to obtain phase shift and relative demodulation parameters for calculation of fluorescence lifetime. Incident light (360 nm) was intensity modulated at 30 MHz. For analysis of lifetime heterogeneity, additional modulation frequencies of 18 MHz and 6 MHz were used. Light emitted from the sample cuvette was detected through a Schott KV-389 cut on filter. The emission beam from the reference chamber, containing rhodamine, was passed through an RG-630 cut on filter. Lifetime calculations were based

upon a reference standard of known lifetime (1.45 ns), consisting of dimethyl POPOP dissolved in ethanol. Phase-modulation theory for computation of fluorescence lifetime and the use of a reference standard in this process has been described [54,55].

Measurements of fluorescence intensity of liposome-incorporated chlorophyll *a* were made with an SLM 8000 fluorometer. Excitation and emission monochromaters were set at 420 nm and 670 nm, respectively. Background was subtracted using blanks consisting of unlabeled liposomes either with or without pyrethroids, as appropriate for experimental and control samples. Instrument fluctuations were internally corrected as described above using rhodamine.

All pyrethroids were dissolved in dimethylsulfoxide prior to addition to cuvettes containing 3 ml of liposomal suspension. Control samples received equivalent aliquots of dimethylsulfoxide. The concentration of dimethylsulfoxide was less than 1% (v/v). For experiments in which pyrethroid concentration was varied, fluorescence measurements were made 5 min after each addition, and temperature was maintained at 25°C by a circulating water bath. For experiments in which temperature was varied, the samples were incubated for 5 min following each change of temperature.

Heterogeneity analysis of diphenylhexatriene fluorescence lifetimes. The χ^2 procedure [56,57] was used to analyze systems of heterogeneous diphenylhexatriene fluorescence lifetimes. Lifetimes were calculated from phase shift and relative modulation parameters at 6 MHz, 18 MHz, and 30 MHz, and the resulting data points were fit to a two component system. The phase angles and fractional fluorescence intensities of two theoretical components were systematically varied until the closest fit to the data was obtained. This best fit was determined by minimization of the statistical parameter, χ^2 [56,57].

Determination of liposome size. To obtain a size estimate of the liposomes used in this study, a sample was prepared for characterization by quasi-elastic light scattering (QELS). DPPC was dried and suspended at a concentration of 5 μM in distilled water. QELS measurements were made using an LDC/Milton-Roy (Chromatix) KMX-6 low-forward angle (4.5 degrees) light scattering photometer. Incident light (632.8 nm) was supplied by a 2 mW He-Ne laser. The DPPC suspension was filtered through a 0.8 μm-pore Metricel membrane filter upon injection into the sample chamber. Photon count data acquisition, storage, and analysis was carried out as previously described [58,59].

Results

Fig. 1 shows the autocovariance function which was obtained from autocorrelation of photon count data obtained by QELS. Nonlinear regression of this function indicated that the DPPC suspension consisted of liposomes with a *z* average Stokes radius of 993 Å.

The concentration-dependent effects of permethrin on fluorescence anisotropy and lifetime of diphenylhexatriene in the DPPC system are shown in Fig. 2. Permethrin decreased anisotropy at concentrations as low as 1 μM, and a pronounced decrease in anisotropy was observed at 100 μM permethrin. Diphenylhexatriene fluorescence lifetime was also decreased over this same permethrin concentration range.

This experiment was repeated for the compound, cypermethrin (data not shown). Although

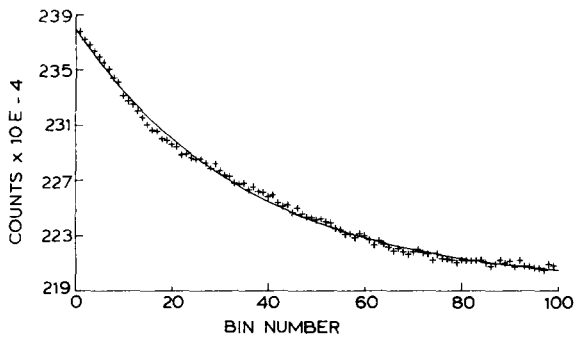

Fig 1. Autocovariance function for DPPC liposomes Liposomes were suspended in distilled water at a concentration of 5 μM. Photons were counted by QELS, at a scattering angle of 4.5 degrees, for 16000 counting intervals, each of 5 ms duration The photon counts were autocorrelated following elimination of intervals which were representative of the transient presence of larger contaminating particles in the scattering volume. The resulting correlated counts were plotted as a function of bin number, with each bin representing a delay time of 5 ms. The data points were fit by nonlinear regression to a single-component exponential decay funcion.

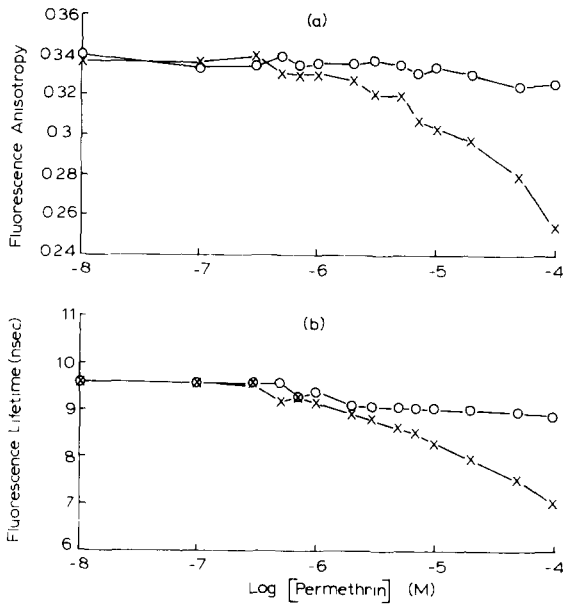

Fig 2 Concentration-dependent effects of permethrin on fluorescence anisotropy (a) and lifetime (b) of diphenylhexatriene in DPPC liposomes at 25°C. DPPC liposomes were labeled with diphenylhexatriene at a lipid/probe molar ratio of 500 1, and were suspended in phosphate-buffered saline at a concentration of $6 \cdot 10^{-5}$ M Permethrin (×) was added in aliquots of dimethylsulfoxide to the liposomal suspension, and the control (○) received equal volumes of dimethylsulfoxide, alone Fluorescence lifetimes were calculated from relative modulation, using a modulation frequency of 30 MHz

anisotropy appeared to decrease slightly near 1 μM cypermethrin, higher concentrations of this compound (near 100 μM) were required to cause a more substantial decrease. Even at 100 μM cypermethrin, the decrease in diphenylhexatriene anisotropy was small in comparison to that detected in the presence of permethrin. Cypermethrin, however, did decrease diphenylhexatriene lifetime in a concentration-dependent manner which was similar to that observed for permethrin. Cypermethrin is the cyano derivative of permethrin, and differs structurally from permethrin only by this cyano substituent. Fenpropathrin and fluvalinate, which are also cyano pyrethroids, were observed to be similar to cypermethrin with regard to their concentration-dependent effects on diphenylhexatriene anisotropy and lifetime. The effects of the cyano pyrethroids on diphenylhexatriene lifetime and anisotropy are consistent with collisional quenching of diphenylhexatriene fluorescence, as such quenching will shorten fluorescence lifetime and increase anisotropy values. Diphenylhexatriene fluorescence lifetime may also be shortened by disruption of lipid packing order, independent of quenching [60]. It is evident that pyrethroids may shorten the fluorescence lifetime of diphenylhexatriene by both quenching and lipid disordering; however, the relative contribution of these two factors may vary between compounds. Cyano pyrethroids appear to quench diphenylhexatriene fluorescence in DPPC liposomes to a greater extent than do pyrethroids not containing the cyano substituent. Results of similar experiments performed in murine splenic lymphocytes [70] are also supportive of such differential quenching capabilities.

Effects of the pyrethroids (10 μM) on the phase transition of DPPC liposomes, as measured by diphenylhexatriene fluorescence anisotropy, are shown in Fig. 3. The control sample demonstrated a sharp transition from the gel to fluid state at 42.5°C, which is near the phase transition temperature of DPPC as detected with diphenylhexatriene anisotropy by other investigators

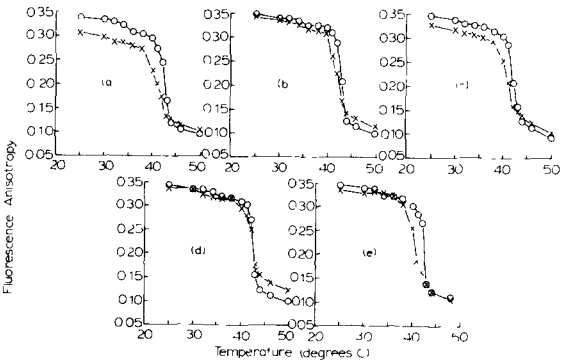

Fig 3 Effects of 10 μM permethrin (a), cypermethrin (b), fenpropathrin (c), fluvalinate (d), and allethrin (e) on the phase transition of DPPC liposomes as determined by diphenylhexatriene fluorescence anisotropy DPPC liposomes were labeled with diphenylhexatriene at a lipid/probe molar ratio of 500 1, and were suspended in phosphate-buffered saline at a concentration of $6 \cdot 10^{-5}$ M Pyrethroids (×) were added in dimethylsulfoxide to the liposomal suspension, and controls (○) received an equal volume of dimethylsulfoxide, alone A 5 min incubation was allowed following each change of temperature

[23,61–63]. The pretransition of DPPC was not detected by a change in diphenylhexatriene anisotropy. The relative insensitivity of diphenylhexatriene to the pretransition of phosphatidylcholines is not unexpected, considering the location of the probe in the hydrocarbon interior of the bilayer [61]. Compared to the control, pyrethroids tended to lower the temperature of phase transition and broaden the temperature range over which this transition occurs. Fluvalinate was least effective in altering the phase transition temperature range (Fig. 3d).

At temperatures well below that of DPPC phase transition, the pyrethroids had differential effects on diphenylhexatriene anisotropy. These results are best interpreted in conjunction with fluorescence lifetime measurements (Fig. 4). Permethrin (Fig. 4a) and fluvalinate (Fig. 4b) both shortened diphenylhexatriene lifetime below the phase transition temperature. However, while permethrin substantially lowered diphenylhexatriene anisotropy at these temperatures (Fig. 3a), fluvalinate had little effect (Fig. 3d). The results again indicate that the relative contribution of lipid disordering and collisional quenching factors to the shortening

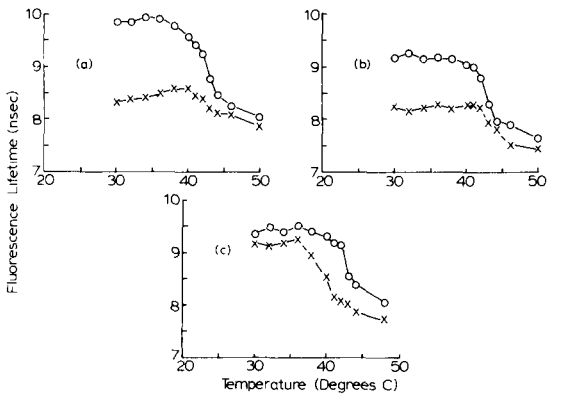

Fig. 4 Effects of 10 μM permethrin (a), fluvalinate (b), and allethrin (c) on the phase transition of DPPC liposomes as determined by diphenylhexatriene fluorescence lifetime. DPPC liposomes were labeled with diphenylhexatriene at a lipid/probe molar ratio of 500 : 1, and were suspended in phosphate-buffered saline at a concentration of $6 \cdot 10^{-5}$ M. Pyrethroids (×) were added in dimethylsulfoxide to the liposomal suspension, and controls (○) received an equal volume of dimethylsulfoxide, alone. Fluorescence lifetimes were calculated from relative modulation, using a modulation frequency of 30 MHz. A 5 min incubation was allowed following each change of temperature.

of fluorescence lifetimes may vary between pyrethroids. The pronounced quenching of diphenylhexatriene fluorescence by fluvalinate is also exemplified by the higher anisotropy readings, relative to control, at temperatures above that of the phase transition. The other cyano pyrethroids, cypermethrin and fenpropathrin, were more similar to permethrin in their effects on the phase transition of DPPC, but were less effective than permethrin in lowering anisotropy below the transition temperature. Collisional quenching of diphenylhexatriene fluorescence in gel phase DPPC by cypermethrin and fenpropathrin would shorten diphenylhexatriene fluorescence lifetime, as was observed (data not shown), and would attenuate a decrease in anisotropy at these lower temperatures. Allethrin, a nonphenoxybenzyl pyrethroid, is interesting in that it had little effect on diphenylhexatriene anisotropy (Fig. 3e) and lifetime (Fig. 4c) at temperatures below that of phase transition. However, allethrin appeared most effective in altering the phase transition temperature range of DPPC.

Heterogeneity analysis of diphenylhexatriene fluorescence lifetimes in gel phase DPPC liposomes was performed. The best fits which could be obtained using three modulation frequencies (6 MHz, 18 MHz, and 30 MHz) indicated that permethrin caused a decrease in the average diphenylhexatriene fluorescence lifetime by shortening the lifetime of the main diphenylhexatriene population. Only approximately 8% of fluorescence intensity was attributable to an additional short (1.4 ns) lifetime component which appeared after exposure to permethrin. The shorter fluorescence lifetimes of diphenylhexatriene in the presence of permethrin may be attributed to the general shortening of lifetimes in the bulk population of diphenylhexatriene molecules, rather than to the generation of a new, distinct population of diphenylhexatriene molecules with much shorter fluorescence lifetimes.

The temperature-dependence of DPPC-incorporated chlorophyll *a* fluorescence intensity was determined. Considering that chlorophyll *a* is likely to be located in the bilayer at the lipid-aqueous interface [49], it follows that the pretransition of DPPC is prominently detected by chlorophyll *a* fluorescence [49,50]. In our study, the fluorescence

intensity of chlorophyll *a* was sensitive only to the pretransition of DPPC. This pretransition was not affected by permethrin at a concentration of 50 μM. Additionally, it appears that the decrease in lipid packing order produced by permethrin is not analogous to enhancement of lateral diffusional freedom in the polar headgroup regions. An increase in lateral diffusive processes produced by the presence of pyrethroids would augment the appearance of the fluorescent, monomeric chlorophyll *a*.

Discussion

Diphenylhexatriene is a useful fluorescent probe for studying alterations in membrane lipid packing order. This fluorophore is an elongated molecule which is located in the interior of the bilayer, and is preferentially oriented with its long axis parallel to the fatty acyl side chains of phospholipids [61]. When the packing order of the hydrocarbon chains is disrupted, the rotation of the diphenylhexatriene molecule between these chains becomes less hindered, resulting in a decreased anisotropy [64–67]. Decreased lipid packing order also results in shortening of the fluorescence lifetime of diphenylhexatriene [60]. The lifetime of a fluorescent probe may also be shortened by collisional quenching [68], which requires the localization of the quencher in the region of the probe [69].

At 25°C, all of the tested pyrethroids with a phenoxybenzyl alcohol shortened the fluorescence lifetime of diphenylhexatriene in a similar manner. These results indicate the localization of these phenoxybenzyl pyrethroids near diphenylhexatriene in the hydrocarbon interior of the DPPC bilayer. Anisotropy measurements revealed disordering of hydrocarbon packing in the presence of permethrin at concentrations above 1 μM. Also supportive of hydrocarbon disordering by permethrin was the shortening of diphenylhexatriene fluorescence lifetimes in gel phase DPPC liposomes. As determined by heterogeneity analysis permethrin shortened the lifetime of the bulk population of diphenylhexatriene molecules. Although a small population of diphenylhexatriene molecules which were characterized by a very short lifetime (1.4 ns) was also detected in the presence of permethrin, definition of this population and resolution of other populations is difficult given the usual limitation of three modulation frequencies (6 MHz, 18 MHz, and 30 MHz).

Cyano pyrethroids required somewhat higher concentrations, relative to permethrin, to cause a pronounced decrease in diphenylhexatriene fluorescence anisotropy. Apparently, the cyano substituent on the phenoxybenzyl moiety enhances the quenching properties of pyrethroids. In similar experiments using diphenylhexatriene-labeled murine splenic lymphocytes, cyano pyrethroids were observed to cause large increases in diphenylhexatriene anisotropy which were not observed in the presence of permethrin [70].

Collisional quenching impaired the ability to detect concentration-related perturbation of gel phase DPPC lipid packing order by cyano pyrethroids at a constant temperature. However, such perturbations were evidenced by the effects of these compounds on the phase transition of DPPC. At 10 μM, the pyrethroids lowered the temperature of DPPC phase transition, and broadened the temperature range over which this transition occurred. Fluvalinate was least effective in altering the phase transition of DPPC. Permethrin and cypermethrin had similar effects on the transition of DPPC from the gel to liquid phase. Since cypermethrin differs from permethrin only in that it is a cyano pyrethroid, it may be concluded that the cyano substituent enhances diphenylhexatriene fluorescence quenching, but does not substantially alter lipid disordering properties. At temperatures below that of DPPC phase transition, permethrin caused the greatest decrease in diphenylhexatriene fluorescence anisotropy. Such decreases in anisotropy were likely to be attenuated in the presence of cyano pyrethroids due to collisional quenching of diphenylhexatriene fluorescence. Collisional quenching was not a factor in the case of the nonphenoxybenzyl pyrethroid, allethrin. Although allethrin effectively altered the phase transition of DPPC, this compound had little effect on diphenylhexatriene lifetime and anisotropy below the transition temperature. One possible explanation for this phenomenon is that allethrin requires higher temperatures in order to solubilize into the DPPC bilayer. Another possibility is that allethrin is present in the bilayer at the lower temperatures, but does not disorder hydrocarbon packing unless

temperatures near that causing phase transition are attained. It is interesting that the relative effects of pyrethroids on DPPC phase transition approximate their relative inhibitory effects on the mitogenic responsiveness of murine splenic lymphocytes to concanavalin A stimulation [71].

In order to examine pyrethroid-induced alterations in the structure of polar headgroups and lateral diffusibility of membrane components near the surface of the bilayer, the fluorescence intensity of membrane-associated chlorophyll *a* was measured in the presence of 50 μM permethrin. Chlorophyll *a*, unlike diphenylhexatriene, is very sensitive to the pretransition of DPPC. Disordering of polar headgroups by permethrin was not detected, as no alterations in the pretransition temperature profile of DPPC were observed. In addition, the fluorescence intensity of chlorophyll *a* was not increased by permethrin at any temperature. These results indicate that disordering of hydrocarbon packing by permethrin does not lead to observable effects in the polar headgroup regions.

In conclusion, pyrethroids have been shown to localize within the hydrocarbon core of DPPC liposomal bilayers. Such localization may be important in determining the availability of pyrethroids to membrane components, such as sodium channels and ATPases. Additionally, pyrethroids were observed to have membrane disordering properties which appeared to be confined to the interior regions of the bilayer. These disordering effects occur at pyrethroid concentrations which are quite similar to, for example, pyrethroid affinity constants for binding sites of pharmacological relevance [40–43] and pyrethroid concentrations which inhibit ATPase activity [45]. Disordering of membrane lipid packing may be a contributory factor to the biological actions of pyrethroids.

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