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The conformation of pyrethroids bound to lipid bilayers

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A number of pyrethroids have been synthesised containing bromine or iodine atoms in the acid and alcohol moieties. Quenching of the fluorescence of a pyrene-containing fatty acid in phospholipid bilayers has been used to measure the partitioning of the pyrethroids into the bilayers. Comparison of the intensity of the fluorescence of the 3-phenoxybenzyl moiety of the pyrethroids in lipid bilayers with that in organic solvents shows that the 3-phenoxybenzyl moiety is located in a hydrophobic environment; this was confirmed by fluorescence quenching by spin-labelled fatty acids. Self-quenching of the fluorescence of pyrethroids which contain both a bromine-containing acid and a 3-phenoxybenzyl moiety is consistent with a 'horseshoe' conformation for the bound pyrethroid, with the ester group located at the lipid/water interface and the acid and alcohol moieties folded back with both penetrating into the lipid bilayer.

Introduction

The pyrethroids are a group of hydrophobic esters with structures based on the natural pyrethrins found in the flowers of the *Chrysanthemum* species and are one of the most commercially important classes of insecticide [1]. The potency of the pyrethroids varies markedly with chemical structure, and, although no individual feature of the molecule appears to be an absolute requirement, high insecticidal activity is usually associated with an ester linkage between an aromatic alcohol and an acid containing a gem dimethyl group [2]. As well as conformational features, hydrophobicity is important, the pyrethroids being more hydrophobic than other classes of insecticide [3,4]. The high hydrophobicity suggests that the site of action of the pyrethroid might be in the biological membrane and, indeed, their primary site of action is thought to be the Na-channel, since they have been shown to drastically prolong the Na current in both vertebrate and invertebrate nerve membranes [1]. It has proved difficult to identify directly the proposed binding sites of the pyrethroid on the Na-channel because the hydrophobic character of the pyrethroid results in very high levels of non-specific binding to biological membranes [5]. Binding to Na-channels has, however, been detected indirectly through effects on the binding of batrachotoxin to the channels, and binding constants of approx. 1–10 μM were obtained [5,6]. Surprisingly, despite the marked stereospecificity of the effects of pyrethroids on the Na-channel [6] it was suggested that DDT binds to the same site as the pyrethroid on the Na-channel [5,7]. As well as Na channels, a number of other membrane proteins have been shown to be affected by pyrethroids at concentrations comparable to those affecting Na-channels, including the GABA receptor [8], the acetylcholine receptor [9] and (Ca^{2+}-Mg^{2+})-ATPase [10–12].

In analysing binding of pyrethroids to membrane proteins, it is useful to understand binding to the lipid component of the membrane, for two reasons. First, for very hydrophobic molecules like the pyrethroids, binding to the lipid component of the membrane will be extensive and thus binding to any specific sites on membrane proteins will be in competition with this non-specific binding. Second, potential binding sites for hydrophobic molecules on membrane proteins exist at the lipid/protein interface, and binding at this interface will be determined by the nature of the binding to the lipid bilayer [13]. Crystal structures of pyrethroids show essentially linear conformations although, in some, flexibility of the phenoxybenzyl moiety gives a more compact structure [14]. Such a conformation would,

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Abbreviations: BrPC, 1,2-bis(9,10-dibromostearoyl)phosphatidylcholine; DCCD, dicyclohexylcarbodiimide; DOPC, dioleoylphosphatidylcholine; PDA, pyrene-1-dodecanoic acid.

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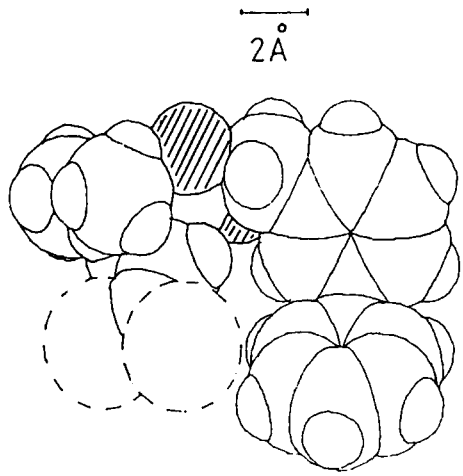

Fig. 1. The structure of *cis*-permethrin in a 'horseshoe' conformation suitable for binding to lipid bilayers, with dashed lines representing chlorine atoms and hatched areas oxygen atoms.

however, be relatively unfavourable for binding to a lipid bilayer. It seems unlikely that the relatively polar ester group of the pyrethroid could be buried in the hydrophobic interior of the lipid bilayer, and there is good experimental evidence that a variety of hydrophobic acids, alcohols and esters are oriented in the membrane with polar groups at the lipid/water interface [15,16]. A linear structure with the ester group at the lipid/water interface would result in either the acid or alcohol moieties extending into the aqueous phase, and this would seem unlikely because of their hydrophobicity. We have therefore suggested [17] that the pyrethroids could adopt the 'horseshoe' conformation illustrated in Fig. 1, with the ester linkage maintained in the energetically favoured transoid conformation, but with both the hydrophobic acid and alcohol moieties folded back to allow penetration into the bilayer; calculations of the relative energies of such a conformation in terms of overlap of Van der Waal's radii show that it is quite feasible (Heritage, K.J., unpublished observations). In this paper we present direct experimental evidence in favour of such a conformation for the pyrethroids.

Materials and Methods

Lipids were obtained from Lipid Products, spin-labelled fatty acids were from Aldrich and pyrene-1-dodecanoic acid (PDA) was from Calbiochem. 1,2-bis(9,10-dibromostearoyl)phosphatidylcholine (BrPC) was prepared as described in Ref. 16.

Preparation of (R,S)-cis,trans-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylic acid

2,2-Dimethyl-3-formylcyclopropanecarboxylic acid methyl ester was prepared from 2,2-dimethyl-3-dimethoxymethylcyclopropanecarboxylic acid methyl ester (1 : 1 mixture of *trans* and *cis* isomers; Dynamit Nobel Chemikalien) by the method of Elliott et al. [18]. The starting material (40 g) was mixed with glacial acetic acid/acetone/water (560 ml; 120 : 160 : 280, v/v) and stirred overnight under nitrogen. The mixture was diluted with excess water and neutralised with saturated sodium carbonate solution. The product was extracted into diethyl ether, dried with magnesium sulphate and purified by vacuum distillation.

(*R,S*)-*cis,trans*-3-(2,2-Dibromovinyl)-2,2-dimethylcyclopropanecarboxylic acid methyl ester was prepared from 2,2-dimethyl-3-formylcyclopropanecarboxylic acid methyl ester by a Wittig reaction as described by Corey and Fuchs [19]. Triphenylphosphine (14.5 g), zinc powder (3.6 g) and dichloromethane (120 ml) were stirred under nitrogen at 0 °C. Carbon tetrabromide (18.3 g) in dichloromethane was added with stirring over 30 min. The mixture was stirred for a further 2 h to ensure complete formation of the ylid. 2,2-Dimethyl-3-formylcyclopropanecarboxylic acid methyl ester (8 g) in dichloromethane (60 ml) was then added slowly. The reaction was allowed to continue overnight. The solvent was removed under reduced pressure and petroleum ether (40–60 °C; 200 ml) was added to form a solid which was filtered off. The solid was ground up and extracted with diethyl ether. The extracts were evaporated, and the residue purified on a Kieselgel H-60 silica gel column eluting with dichloromethane. The product was redissolved in *n*-hexane and diethyl ether (12 : 1, v/v) and the *cis* and *trans* esters separated by HPLC (Gilson Model 302, silica gel column).

The free acid was prepared by hydrolysis of the methyl ester with sodium hydroxide in ethanol, followed by crystallisation from petroleum ether (60–80 °C).

Preparation of 3-(4-iodophenoxy)bromomethylbenzene

3-(4-Iodophenoxy)toluene was prepared by an Ullmann ether coupling reaction between 1,4-diiodobenzene and *m*-cresol. *m*-Cresol (13 g) in dimethylformamide (80 ml) was added dropwise over 30 min to a stirred suspension of sodium hydride (4.8 g) in dimethylformamide (100 ml), keeping the temperature below 45 °C. The reaction was continued for a further 1 h, then cuprous chloride (9.9 g) and 1,4-diiodobenzene (40 g) were added and the mixture heated at 100 °C for 1.5 h and then at 120 °C for 2.5 h. The reaction mixture was filtered and the solvent removed by evaporation. The residue was taken up in ethyl acetate (150 ml) and extracted successively with 2 M HCl and water. The product, 3-(4-iodophenoxy)toluene, was purified on a silica gel column eluting with *n*-hexane/diethyl ether (40 : 1, v/v).

3-(4-Iodophenoxy)toluene (3.1 g) and *N*-bromosuccinimide (1.8 g) were added to carbon tetrachloride (60 ml). A trace of dibenzoyl peroxide was added, the mixture heated until it started to reflux, and then il-

luminated with a 140 W bulb. After 3 h *N*-bromosuccinimide (0.9 g) was added and the reaction continued for a further 4 h. The carbon tetrachloride was evaporated off, and the mixture dissolved in diethyl ether, washed with a saturated aqueous solution of sodium thiosulphate followed by water, and finally dried over magnesium sulphate. The diethyl ether was removed, and the mixture dissolved in a small amount of *n*-hexane/dichloromethane (12 : 1, v/v) before purification on a silica gel column.

Preparation of pyrethroids

Coupling of acids and alcohols was performed as follows. To the acid (1 mol equivalent) and a trace of 4-dimethylaminopyridine in dichloromethane was added dicyclohexylcarbodiimide (1 mol equivalent) and 1.05 mol equivalent of the alcohol in dichloromethane. The mixture was allowed to stand at room temperature overnight. Dicyclohexylurea was then removed by filtration. The solution was concentrated and the product purified on a silica gel column eluting with dichloromethane. The esters formed single spots on TLC with R_f value of 0.7–0.8.

3-(4-Iodophenoxy)bromomethylbenzene was esterified as follows. The acid (1 mol equivalent), 3-(4-iodophenoxy)bromomethylbenzene (1 mol equivalent) and potassium carbonate (1.25 mol equivalent) were stirred in acetone at room temperature overnight. The mixture was filtered, and the acetone removed by evaporation. The residue was redissolved in diethyl ether/*n*-hexane (1 : 10, v/v) and purified on a silica gel column.

All compounds were characterised by GC, TLC, IR, NMR and mass spectrometry and shown to be > 95% pure. Structures of the pyrethroids used in this study are given in Fig. 2.

Fluorescence spectroscopy

Fluorescence spectra were recorded with a Perkin-Elmer MPF44A fluorimeter. In studies of the effects of pyrethroids on the fluorescence of pyrene-1-dodecanoic acid (PDA) in liposomes, dioleoylphosphatidylcholine (DOPC) and PDA (molar ratio 500 : 1) were mixed in chloroform solution, dried onto the sides of flasks, and resuspended in buffer (40 mM Hepes, 100 mM NaCl, 1 mM EGTA, pH 7.2). Pyrethroids were either added to the original chloroform mixture or added to the liposomes as a concentrated solution in dimethylformamide followed by a 5 min equilibration: the final concentration of dimethylformamide did not exceed 0.3%. Comparison of the results of 5 min and over-night equilibrations showed that the former was sufficient for equilibrium to be achieved. Control samples were prepared as above but in the absence of PDA, so that corrections could be made for light scatter. PDA fluorescence was excited at 342 nm and observed at 395 nm.

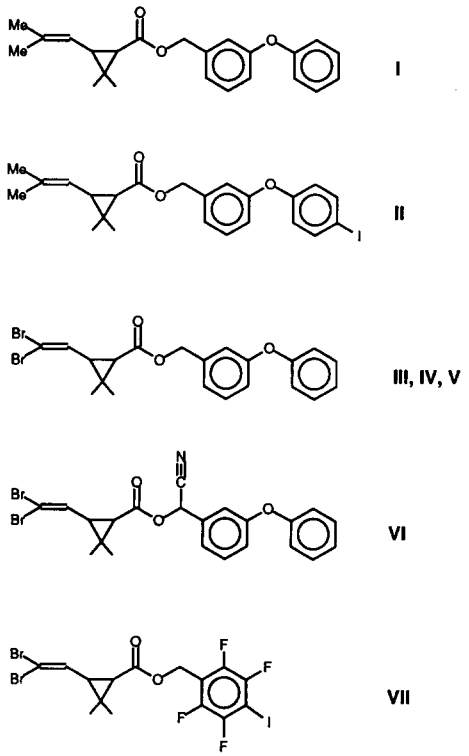

Fig. 2. Structures of pyrethroids studied: I, *cis*: *trans* 3:7 (*R*,*S*); II, *cis*: *trans* 3:7 (*R*,*S*); III, *cis* (*R*,*S*); IV, *trans* (*R*,*S*); V, *cis* (1*R*); VI, *cis* (1*R*α*S*) (deltamethrin); VII, *cis* (*R*,*S*).

Quenching of the fluorescence of the pyrethroids in liposomes by spin-labelled fatty acids was observed by drying DOPC (245 μM) and pyrethroid (122 μM) in chloroform, followed by formation of liposomes as described above. Spin-labelled fatty acids were added to the liposomes and equilibrated for up to 10 min before measurement of fluorescence intensity. The concentrations of spin-labelled fatty acids were calibrated by comparison of the second derivatives of ESR spectra recorded in methanol on a Bruker ER 200D ESR spectrometer.

Analysis of quenching data

Quenching of fluorescence in a lipid bilayer may be described by the Stern-Volmer equation:

$$F_o/F = 1 + K_{sv}Q_m \quad (1)$$

where F_o and F are fluorescence intensities in the absence and presence of quencher respectively, Q_m is the concentration of quencher in the membrane and K_{sv} is the quenching constant. Quenching in these systems is likely to be dynamic (see Ref. 23) but static quenching would give an equation for quenching of exactly the same form as Eqn. 1 [26].

In the partition model, the distribution of quencher between membrane and aqueous phases is given by a partition coefficient K_p, where:

$$K_p = Q_m/Q_a \quad (2)$$

and Q_m and Q_a are concentrations of quencher in the membrane and aqueous phases, respectively, expressed in units of moles per litre. Since the total number of moles of quencher added must equal the sum of those in the aqueous and membrane phases:

$$V_t Q_t = V_m Q_m + V_a Q_a \quad (3)$$

where V_t, V_m and V_a are the total volumes and the volumes of the membrane and aqueous phases respectively, and Q_t is the total concentration of quencher added. Quenching can then be expressed as:

$$F_o/F = 1.0 + [K_{sv} K_p V_t Q_t]/[V_a + K_p V_m] \quad (4)$$

In the calculation of V_m, the vesicle density was taken as 1 g/ml [22]. Following Omann and Lakowicz [23], the volume fraction α_m of the membrane can be defined as:

$$\alpha_m = V_m/V_t \quad (5)$$

Substitution into Eqn. 4 gives:

$$F_o/F = 1.0 + [K_{sv} K_p Q_t]/[K_p \alpha_m + (1 - \alpha_m)] \quad (6)$$

At any particular lipid concentration α_m, K_p and K_{sv} are all constant and an apparent quenching constant K_{app} can be defined as:

$$K_{app} = K_p K_{sv}/[K_p \alpha_m + (1 - \alpha_m)] \quad (7)$$

Eqn. 6 then simplifies to:

$$F_o/F = 1.0 + K_{app} Q_t \quad (8)$$

A graph of F_o/F against Q_t will be a straight line with slope K_{app}. From Eqn. 7 a plot of $1/K_{app}$ against α_m will also be a straight line, with a slope given by:

$$\text{slope} = (1/K_{sv}) - (1/K_p K_{sv}) \quad (9)$$

and an intercept given by:

$$\text{intercept} = 1/K_p K_{sv} \quad (10)$$

so that

$$K_p = \text{slope}/\text{intercept} \quad (11)$$

and

$$1/K_{sv} = \text{slope} + \text{intercept} \quad (12)$$

An alternative to the partition model is to describe binding by a Langmuir adsorption isotherm [24]. In the binding model, the maximum extent of binding to the bilayer is limited, the limit being expressed either in terms of the maximum number of molecules N adsorbed per phospholipid molecule or in terms of the maximum number of molecules adsorbed per unit area. Binding can be expressed in terms of a 'site' concentration given by NE_t, where E_t is the total lipid concentration. The concentration of bound quencher Q_b is given by:

$$Q_b = Q_a E_f/K_d \quad (13)$$

where E_f is the free site concentration and K_d is the dissociation constant for binding. In terms of total concentrations:

$$Q_b = \left(A - [A^2 - 4NE_t Q_t]^{1/2}\right)/2.0 \quad (14)$$

where

$$A = K_d + NE_t + Q_t \quad (15)$$

Fluorescence quenching can then be expressed as:

$$F_o/F = 1.0 + K'_{sv} Q_b/(NE_t) \quad (16)$$

The Stern-Volmer constant in Eqn. 16 is different to that in Eqn. 1 because of the different concentration units. In the region where only a small fraction of the lipid sites are occupied, it can be shown that the two constants are related by:

$$K'_{sv} = K_{sv} N V_t E_t/V_m \quad (17)$$

Further, in this region, K_p is related to K_d by:

$$K_d = 1000 \cdot N/(K_p m \rho_l) \quad (18)$$

where m and ρ_l are the molecular weight and density (g/ml) of the lipid, respectively.

In some systems it has been found that fluorescence quenching data plotted according to the Stern-Volmer relationship shows upward curvature, and it has been suggested that this arises because only a fraction of the fluorophores are quenched by a collisional mechanism, the rest being quenched by a static mechanism. In such cases quenching can often be described by a modified Stern-Volmer equation:

$$F_o/F = [1.0 + K_{sv} Q_m] \exp(V Q_m) \quad (19)$$

where V is a static quenching constant [25].

Results

Molecules containing bromine and iodine are frequently efficient quenchers of fluorescence, and fluorescence quenching can be used in a variety of ways to study interaction of hydrophobic molecules with lipid bilayers (see review in Ref. 26). We have therefore synthesised a number of pyrethroids containing bromine

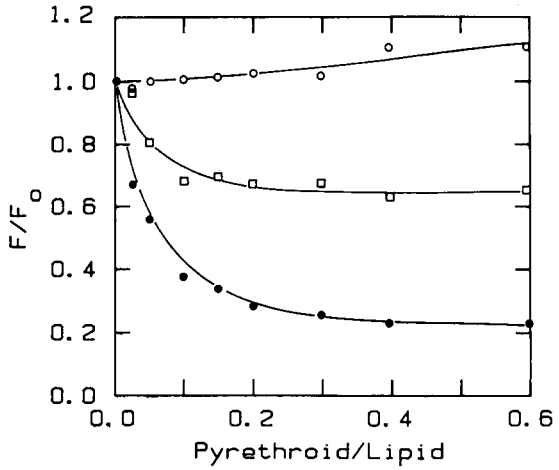

Fig. 3. Fluorescence quenching (F/F_o) of PDA in liposomes of DOPC (41 μM) at 37°C, on addition of pyrethroids at the given molar ratios of pyrethroid to phospholipid; ○, I; □, III; ●, II. The molar ratio of PDA to DOPC was 1:500.

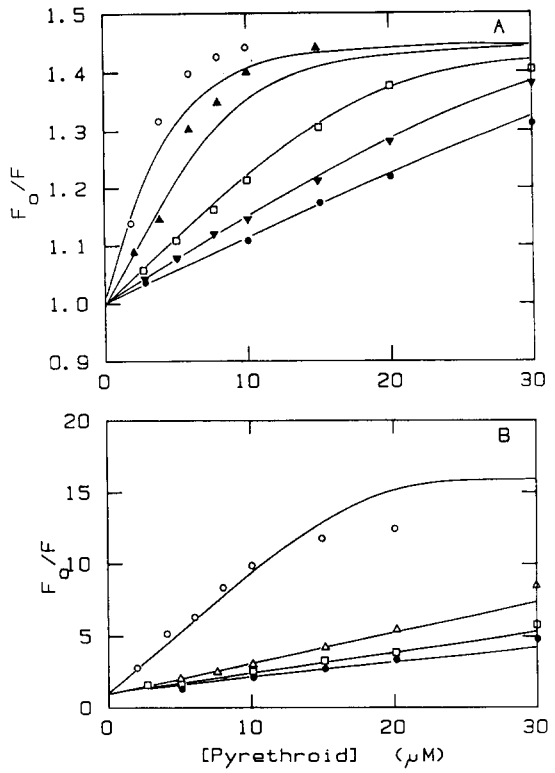

Fig. 4. Fluorescence quenching (F_o/F) of PDA in liposomes of DOPC at 25°C on addition of (A), pyrethroid III and (B), pyrethroid VII at the given aqueous concentrations, at DOPC concentrations (μM) of (A); ○, 50; ▲, 100; □, 200; ▼, 300; ●, 400; and (B); ○, 50; △, 200; □, 300; ●, 400. The curves are quenching plots calculated using the binding model with the parameters given in Table I.

or iodine atoms in either the acid or alcohol moieties (Fig. 2). As a fluorescence probe for the phospholipid bilayer we used pyrene dodecanoic acid (PDA) since this has been shown to bind strongly to phospholipid bilayers with the pyrene group located in the fatty acyl chain region of the bilayer [16]. Fig. 3 shows the effect of pyrethroids on the fluorescence of PDA incorporated into bilayers of DOPC. Addition of pyrethroid I containing no quenching groups had essentially no effect on the intensity of fluorescence emission of PDA. In contrast addition of either pyrethroid III containing two bromine atoms in the acid moiety or pyrethroid II containing an iodine in the alcohol moiety resulted in marked quenching of fluorescence, indicating that both acid and alcohol moieties of the pyrethroid penetrate into the hydrophobic interior of the phospholipid bilayer.

As described in Materials and Methods, studies of quenching as a function of lipid and quencher concentrations can be analysed to give binding constants for the quencher. Fig. 4A shows the quenching of PDA fluorescence by pyrethroid III as a function of lipid concentration. The extent of fluorescence quenching increased with increasing concentration of quencher up to a limiting value, beyond which addition of further quencher had no further effect on fluorescence. Quenching in the initial region where quenching changed linearly with quencher concentration can be analysed in terms of the partition model. A graph of F_o/F against pyrethroid concentration at a fixed lipid concentration gives K_{app}, defined by Eqn. 7. From a plot of $1/K_{app}$ at different lipid concentrations against the volume fraction of the membrane α_m (Fig. 5), it is possible to derive the partition coefficient for the pyrethroid (Eqn. 11). Results obtained in this way are given in Table I. For pyrethroids IV and V, quenching data is comparable to that shown in Fig. 4A, but quenching plots for pyrethroid VII show upward curvature (Fig. 4B). Data for pyrethroid VII was therefore analysed in terms of Eqn. 19, with the results again listed in Table I.

An alternative approach to the analysis of the quenching data is in terms of a binding model, in which binding of the pyrethroid is limited because of a limited number of 'binding sites' within the bilayer (Eqn. 16). As shown in Figs. 4A and B, the quenching data can be analysed over the whole concentration range in terms of

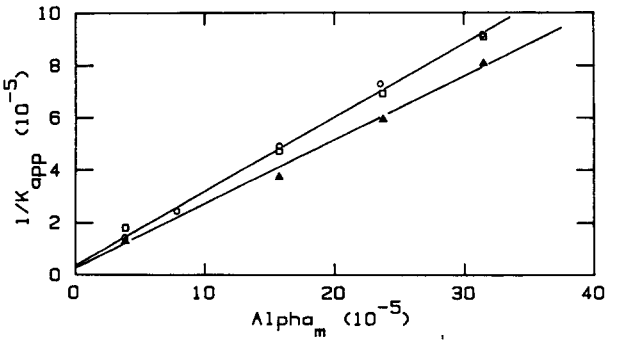

Fig. 5. Plot of $1/K_{app}$ against the membrane volume fraction α_m derived from the partition model described in the text, for quenching of PDA in liposomes of DOPC at 25°C for pyrethroids; ○, III; □, V; ▲, IV.

TABLE I

Binding and Stern-Volmer quenching constants for pyrethroids at 25°C

Pyrethroid	K_p	K_{sv} [a] (M^{-1})	K_d (μM)	N (mol/mol)
III	$1.0 \cdot 10^5$	3.6	0.7	0.1
IV	$1.3 \cdot 10^5$	4.3	1.3	0.1
V	$1.0 \cdot 10^5$	3.6	0.9	0.1
VII	$1.0 \cdot 10^5$	34.4 [b]	0.3	0.35
Methyl ester [c]	$1.2 \cdot 10^4$	2.2	70	0.6

[a] Calculated from partition model (see text).
[b] With static quenching constant ($V = 21.0\ M^{-1}$).
[c] *trans*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylic acid methyl ester.

this model with the results listed in Table I; for the binding model, the Stern-Volmer quenching constant K'_{sv} was calculated from the Stern-Volmer constant K_{sv} obtained from the partition analysis and given in Table I, using Eqn. 17.

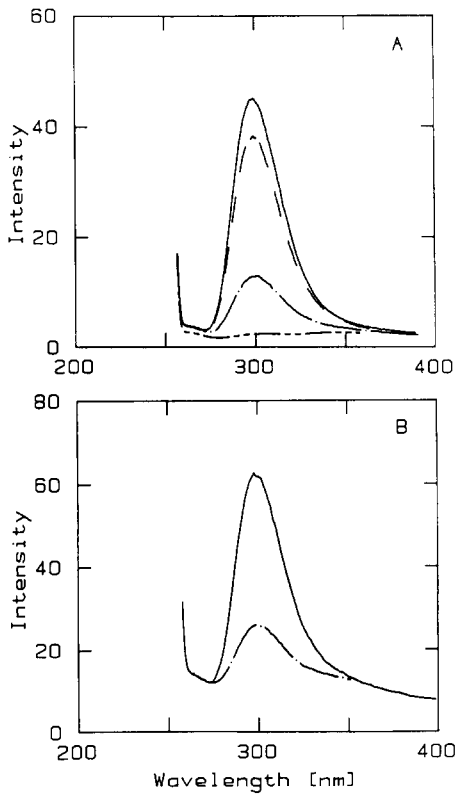

Fig. 6. Fluorescence emission spectra of pyrethroids in DOPC at 25°C, excited at 240 nm. (A). ———, DOPC (200 μM) and pyrethroid I (10 μM); – – –, DOPC (200 μM) and pyrethroid I (10 μM) plus pyrethroid III (10 μM); ·–·–·, BrPC (200 μM) and pyrethroid I (10 μM); – · · –, DOPC (200 μM) alone. The emission spectrum of DOPC (20 μM) and pyrethroid III (10 μM) is identical to the spectrum shown for BrPC (200 μM) and pyrethroid I (10 μM) (·–·–·). (B). ———, DOPC (1 mM) and pyrethroid I (10 μM); ·–·–·, DOPC (1 mM) and pyrethroid III (10 μM). The emission spectrum of DOPC (1 mM) and pyrethroid I (10 μM) plus pyrethroid III (10 μM) is identical to that for DOPC (1 mM) and pyrethroid I (10 μM) (———).

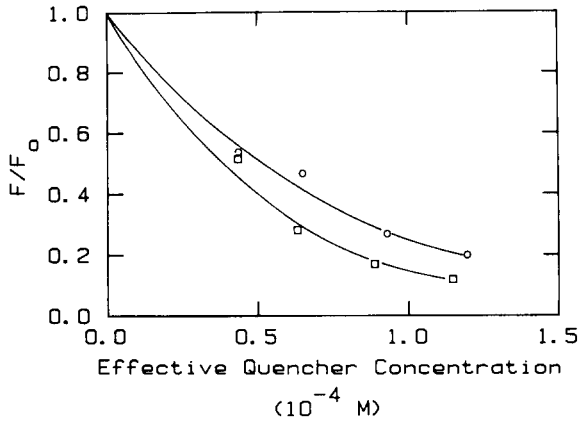

Fig. 7. Fluorescence quenching (F/F_0) of pyrethroid I (122 μM) in DOPC (245 μM) by 5-doxyl (○) and 16-doxyl stearic acids (□). The effective quencher concentrations are the concentrations of bound fatty acid, expressed as moles fatty acid/litre of total sample, calculated as described in the text.

We have found that 3-phenoxybenzyl alcohol is fluorescent, with fluorescence excitation and emission maxima (uncorrected) at 240 and 294 nm, respectively (data not shown). As shown in Fig. 6, pyrethroids containing the 3-phenoxybenzyl moiety are also fluorescent. We found that the emission maximum varied little with environment, but that fluorescence intensities in organic solvents increased significantly with decreasing solvent polarity (Table II). The intensity of fluorescence of pyrethroid I bound to bilayers of DOPC was comparable to that observed in hexane, indicating that the 3-phenoxybenzyl moiety was located in a very hydrophobic environment. This was also shown by quenching of the fluorescence by spin-labelled fatty acids, where the quenching nitroxide group is located within the fatty acyl chain region of the bilayer (Fig. 7). Blatt et al. [20] have shown that binding constants for spin-labelled fatty acids to lipid bilayers differ depending on the positions of the nitroxide group on the fatty acid chain, and this needs to be taken into account in any study of the relative quenching efficiencies of the spin-labelled fatty acids. Rooney et al. [21] have shown how binding

TABLE II

Solvent dependence of fluorescence emission of pyrethroid I

Fluorescence excited at 240 nm and observed at 294 nm.

Solvent	Relative fluorescence intensity [a]	Dielectric constant [b]
Water	0.27	78
Methanol	0.34	33
2-Propanol	0.49	18
Hexane	1.0	1.9
DOPC	0.89	-

[a] Measured relative to that in hexane.
[b] From Ref. 27.

of spin-labelled fatty acids to lipid bilayers can be calculated from binding constants and they determined the binding constant for 12-doxylstearic acid (1.75 μM). From the ratios of the partition coefficients determined by Blatt et al. [20], binding constants for 5- and 16-doxylstearic acids can be estimated to be 0.9 and 2.1 μM, respectively. Binding of these spin-labelled fatty acids to the lipid bilayers were then calculated as described by Rooney et al. [21]. In Fig. 7, quenching of pyrethroid fluorescence by the spin-labelled fatty acids has been plotted against the concentration of bound fatty acid, expressed as moles per litre of sample.

Discussion

Pyrethroids are very hydrophobic and show extensive binding to phospholipid bilayers and to the phospholipid component of biological membranes [5,17,31]. A correlation has been established between hydrophobicity and insecticidal potency [28]. Binding to the phospholipid bilayer has little effect on the fluidity of the bilayer, as long as the phospholipids are in the biologically relevant liquid-crystalline phase [17,31]. Effects of pyrethroids are much more likely to follow from direct interaction with target proteins in the membrane, although the nature of the binding sites has yet to be established. The primary effects of the pyrethroids seem to follow largely from binding to Na-channels [1,5,6], but interaction with a variety of other membrane protiens, including transport ATPases, has been observed [8–12]. We have found that a wide variety of hydrophobic molecules can bind to the (Ca^{2+}-Mg^{2+})-ATPase and affect its activity, and have detected binding sites at the lipid-protein interface (annular sites) and at other hydrophobic sites on the ATPase (non-annular sites) which could be at protein-protein interfaces in aggregates of the ATPase (dimers or rows of monomers or dimers) [11,13,16,17].

The binding of hydrophobic molecules at the lipid-protein interface will depend on the nature of their binding to lipid bilayers. The conformation adopted by the pyrethroids on binding to lipid bilayers is unclear. In crystals, the pyrethroids adopt an essentially linear structure [14]. Simple partition of the pyrethroids in such a conformation into the hydrophobic interior of a phospholipid bilayer seems unlikely, since it would involve the unfavourale location of the polar ester group in a non-polar environment. It has been shown that the ester group in cholesterol esters bound to phospholipid bilayers is located at the lipid/water interface [15]. For the pyrethroids to adopt a linear conformation with the ester group located at the lipid/water interface would mean that, for an individual pyrethroid molecule, either the acid or the alcohol moiety would penetrate into the aqueous phase, the relative proportion of the molecules adopting the two possible orientations depending on the relative hydrophobicities of the acid and alcohol moieties. We suggested [17] an alternative possibility, in which the pyrethroid adopted a 'horseshoe' conformation, in which the ester linkage is maintained in the energetically favoured transoid conformation, but with both the acid and alcohol moieties folded back to allow penetration into the bilayer (Fig. 1).

In this paper we have used fluorescence quenching methods to study the conformation of the pyrethroids bound to phospholipid bilayers. We have synthesized a number of pyrethroids containing bromine or iodine in the acid and alcohol moieties, related in structure to deltamethrin (Fig. 2), a commercially important pyrethroid. The compounds all show insecticidal activity (unpublished observations).

Quenching of fluorescence by halogens involves close contact between the halogen and the fluorophore and thus can be used to determine relative locations of the halogen and fluorophore [26]. The observation that pyrethroid III with bromines in the acid moiety and pyrethroid II with an iodine in the alcohol moiety can both quench fluorescence of the hydrophobic PDA in lipid bilayers (Fig. 3), shows that both acid and alcohol moieties can penetrate into the hydrophobic interior of the phospholipid bilayer. The greater quenching caused by the iodine-containing pyrethroid is likely to follow from the known greater quenching efficiency of iodine [26]. Further evidence that the alcohol moiety can penetrate into the hydrophobic interior of the bilayer comes from the observation that the fluorescence intensity of the 3-phenoxybenzyl moiety of pyrethroid I bound to lipid bilayers is comparable to that in hexane rather than to that in a polar solvent (Table II) and from the observation that fluorescence is quenched by spin-labelled fatty acids (Fig. 7). Although the mechanism of quenching by nitroxide free radicals is not yet established, it seems to require a collision between the fluorescent group and the nitroxide, in which the two groups come closer than about 10 Å [32,33]. If the pyrethroid is anchored at the lipid/water interface, then the alcohol moiety will penetrate some 12 Å below the glycerol backbone region of the bilayer. Since the thickness of the DOPC bilayer between the glycerol backbone regions is 30 Å [29], the alcohol moiety would be equally accessible to nitroxide groups at either the 5- or the 16-positions of a fatty acid chain, consistent with the very similar quenching observed for 5- and 16-doxyl stearic acids, when expressed in terms of bound concentrations of fatty acid (Fig. 7).

To distinguish between simultaneous binding of acid and alcohol moieties for a pyrethroid in a 'horseshoe' conformation and alternate binding of acid or alcohol moieties in a linear conformation, we studied self quenching of pyrethroid fluorescence in the bilayer (Fig. 6). As described above, the 3-phenoxybenzyl moiety in pyrethroid I is fluorescent, and the fluorescence inten-

sity increses when the pyrethroid partitions from water into bilayers of DOPC (Table II, Fig. 6). On partitioning into bilayers of BrPC, a lipid containing C-Br bonds in its fatty acyl chains, the fluorescence intensity decreases (Fig. 6), showing that bromine atoms quench the fluorescence of this fluorophore. For pyrethroid III which contains the 3-phenoxybenzyl group as well as two bromines in the acid moiety, intramolecular quenching of fluorescence would indicate that the pyrethroid adopts a conformation in which the bromines of the acid portion come into close contact with the 3-phenoxybenzyl moiety, that is, a 'horseshoe' conformation of the type shown in Fig. 1; it is, however, necessary to eliminate the possibility of inter-molecular quenching at the high concentrations of pyrethroid reached within the membrane. Fig. 6A shows that the fluorescence intensity of pyrethroid III in bilayers of DOPC at relatively high molar ratios of pyrethroid to lipid (1 : 20) is low and equal to that observed for pyrethroid I in BrPC, indicating a high level of quenching for pyrethroid III. Relatively little of this quenching is likely to be inter-molecular since addition of pyrethroid III to pyrethroid I in DOPC results in relatively little quenching of fluorescence (Fig. 6A). This is confirmed by experiments at lower molar ratios of pyrethroid to lipid (1 : 100) where addition of pyrethroid III has less effect on the fluorescence of pyrethroid I, but the fluorescence intensity of pyrethroid III is still very low (Fig. 6B). We conclude that the pyrethroid adopts a conformation in the bilayer in which the acid and alcohol moieties come into close contact.

An alternative approach to the study of the bound conformation of the pyrethroids is by comparison of the measured partition coefficients with those expected for binding in linear or 'horseshoe' conformations. Based on fluorescence quenching studies, it is possible to measure partition constants for the pyrethroids from least-squares fits of $1/K_{app}$ vs. α_m (Fig. 5; Table I). For pyrethroid III, for example, a partition coefficient of $1.0 \cdot 10^5$ was obtained, with a standard error giving upper and lower limits of $1.9 \cdot 10^5$ and $0.7 \cdot 10^5$, respectively. The same data can be analysed in terms of a binding model, giving a dissociation constant for binding of 0.7 μM and a number of binding sites of 0.1 per phospholipid molecule (Table I). The results obtained using the two approaches are in good agreement; a partition coefficient of $1.0 \cdot 10^5$ for example, corresponds, from Eqn. 18, to a dissociation constant for binding of 1.4 μM with an N value of 0.1, in good agreement with the value obtained from the analysis in terms of the binding model (Table I). The measured partition coefficient is a factor of 10 greater than that measured for the methyl ester of the acid moiety (Table I). The partition coefficient for the methyl ester is very close to the value of log $P = 4.07$ estimated for the octanol/water partition coefficient from the parameters of Leo et al. [30]. An octanol/water partition coefficient of log $P = 3.25$ can be estimated for 3-phenoxybenzyl alcohol also from the data of Leo et al. [30]. The observation that the measured phospholipid/water partition coefficients for the pyrethroids are greater than those for either the methyl ester of the acid or the alcohol is consistent with a structure in which both the acid and the alcohol moieties bind to the hydrophobic interior of the bilayer. The measured phospholipid/water partition coefficients for the pyrethroids (Table I) are, however, significantly less than the estimated octanol/water partition coefficients (log $P \approx 8.0$; Pierce, A., unpublished observations), suggesting that a significant portion of the hydrophobic surface of the pyrethroid must be exposed in relatively polar regions when the pyrethroids bind to a phospholipid bilayer, again consistent with binding of the pyrethroid in a 'horseshoe' conformation (Fig. 1) with the ester group located at the phospholipid/water interface. In part, however,the lower phospholipid/water than octanol/water partition coefficient can be attributed to the limited binding of the pyrethroid to phospholipid bilayers, with a maximum binding of approx. 1 pyrethroid per 10 phospholipid molecules (Table I). Limited miscibility has previously been observed in the binding of a variety of hydrophobic molecules to phospholipid bilayers [11,15,16].

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