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Effect of fenvalerate, a pyrethroid insecticide on membrane fluidity

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Fenvalerate is a commonly used pyrethroid insecticide, used to control a wide range of pests. We have studied its interaction with the membrane using fluorescence polarization and differential scanning calorimetry (DSC) techniques. Fenvalerate was found to decrease the DPH fluorescence polarization value of synaptosomal and microsomal membrane, implicating that it makes the membrane more fluid. At different concentrations of fenvalerate, the activation energy of the probe molecule in the membrane also changes revealed from the change in slope of the Arrhenius plot. At higher concentrations the insecticide slowly saturates the membrane. The effects of fenvalerate on model membrane were also studied with liposomes reconstituted with dipalmitoylphosphatidylcholine (DPPC). Fenvalerate decreased the phase transition temperature (T_m) of DPPC by 1.5 C° at 40 μM concentration, but there was no effect on the cooperativity of the transition as interpreted from the DSC thermogram. From the change in the thermogram profile with fenvalerate it has been interpreted that it localizes in the acyl chain region of the lipid, possibly between C_{10} and C_{16} region and weakens the acyl chain packing. Fenvalerate was also found to interact with DPPC liposomes containing cholesterol to fluidize it.

Introduction

Insecticides have been shown to partition into membranes and cause change in membrane fluidity [1]. Extensive studies on interaction of insecticide with membranes have been done for DDT [2,3] and lindane [4,5]. Other types of insecticides have not received similar attention in this respect and details of their membrane interactions are not known.

Fenvalerate (Sumicidin$^{(R)}$, (*RS*)-α-cyano-3-phenoxybenzyl-(*RS*)-2-(4-chlorophenyl)-3-methylbutyrate) is one of the most commonly used pyrethroid insecticides, used to control a wide range of pest insects of cotton and vegetables [6,7]. This insecticide has four optical isomers because of the presence of two asymmetric carbons in the molecule. It has been shown to cause microgranulomatous lesions in adrenal glands, mesenteric lymph nodes, liver and/or spleen of rats and mice at toxic doses [8]. Fenvalerate has also been shown to have considerable effect on the electrical properties of nerve fibre. In the skin sense organ of *Xenopus laevis* it showed intense repetetive activity. It has also been shown to affect the myelinated nerve fibres in a similar way [9]. Fenvalerate depolarizes the membrane of crayfish axon [10] and it also induces a steady-state sodium current at potentials less negative than −100 mV [11,12]. However, it did not affect the potassium current in crayfish axons. Fenvalerate also blocks the GABA-activated conductance in crayfish axon [13].

The main target for fenvalerate as well as other pyrethroids appears to be the ionic channels of biomembranes. But so far direct interaction of fenvalerate with biomembranes have not been reported. Here we report direct interaction of fenvalerate with membranes, both natural and model liposomes. We have monitored the fluidity parameter to see the changes in membrane dynamics due to insecticide interaction by measuring the fluorescence polarization of DPH incorporated into the membrane. As the insecticide mostly affects the nervous tissue we have chosen synaptosomal and microsomal membrane from rat brain in this study.

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; GABA, γ-aminobutyric acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; THF, tetrahydrofuran; DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; lindane, γ-1,3,4,6,2,5-hexachlorocyclohexane.

Materials and Methods

Preparation of natural membranes

The brain synaptosomal membranes were isolated by standard sucrose gradient centrifugation as described [14]. Briefly the whole brain from young male Wistar rats (15-day-old) were removed and homogenized in 9 vol. of 0.32 M sucrose, 5 mM Na-Hepes (Sigma) (pH 7.0). The nuclear pellet was discarded by centrifuging at $2200 \times g$ for 10 min. The resultant supernatant was then loaded on 1/2 vol. of 1.2 M sucrose 5 mM Na-Hepes (pH 7.0) and centrifuged at $200\,000 \times g$ for 15 min. The material at the interface of 1.2 M and 0.32 M sucrose layer was collected, diluted with 0.32 M sucrose and again loaded on equal vol. of 0.8 M sucrose, 5 mM Na-Hepes (pH 7.0) and centrifuged at $200\,000 \times g$ for 15 min. The synaptosomal pellet thus obtained was frozen and stored at −20°C.

The microsomal membrane was prepared by subcellular fractionation [15]. Briefly the whole brain from male wistar rats (15-day-old) was removed and homogenized in 0.25 M sucrose (10% homogenate). The nuclei were removed by spinning the homogenate at $1500 \times g$ for 10 min. The supernatant was then centrifuged at $22\,000 \times g$ for 20 min to remove mitochondria and the supernatant was centrifuged at $102\,500 \times g$ for 45 min to get microsomal pellet. The pellet was frozen and kept at −20°C.

Preparation of DPPC liposomes

DPPC liposomes were made following standard method [16,17]. 5 mg of DPPC (Sigma) was weighed, dissolved in chloroform and the solvent was evaporated in a round bottom flask to form a film of lipid. The lipid film was then hydrated in 0.1 M phosphate buffer (pH 7.0) and dispersed by handshaking in a water bath at about 45°C. The dispersion was then sonicated in pulses for 15 min in a Branson B15 Sonifier. The resulting liposome suspension consisting of heterogeneous mostly uni- and multi-lamellar vesicles were diluted with phosphate buffer to get an OD_{425} of about 0.11 without any centrifugation and used for further fluorescence measurements. Control fluorescence polarization measurements done without probe did not give any appreciable fluorescence polarization values suggesting that there was no light scattering problem at the vesicle concentration used ($OD_{425} = 0.11$) [18,19]. DPPC liposomes were loaded with DPH similar to natural membrane as described below.

DPH and fenvalerate incorporation into membrane

The membranes (synaptosomal/microsomal pellet and DPPC liposomes) were dispersed in 0.1 M phosphate buffer (pH 7.0). The OD_{425} was monitored around 0.11 to get a uniform suspension suitable for fluorescence study. From a stock of 2 mM DPH (Sigma) in tetrahydrofuran (THF), 2 μl was injected for each 2 ml of membrane suspension with vigorous stirring at room temp. [18]. It has been seen that 30 min stirring at room temp. gives an uniform equilibration of probe into the membrane. Fenvalerate (Rallis, India) was added from a concentrated ethanolic stock solution (20 mM). After addition of each aliquot of fenvalerate to the sample a 15 min equilibration time was allowed before the reading was taken.

Fluorescence measurements

The fluorescence spectra of DPH in presence of fenvalerate was taken in a Perkin-Elmer Spectrofluorimeter (model MPF-44A). This was done in order to check whether fenvalerate affects the intrinsic fluorescence property of DPH. The concentrated solution of fenvalerate in THF was directly added to the cuvette containing 2 μM DPH in THF according to required final concentration.

Fluorescence polarization experiments were done on a Union-Giken FS 501 Fluorescence Polarization Spectrophotometer, equipped with photon counting photomultipliers and thermostated sample holder. Samples were excited at 355 nm with a 3.5 nm slit and emission was monitored by means of a bandpass filter ($\lambda_{1/2} = 7$ nm) centered at 428 nm along with a 420 nm cutoff filter. The samples in $1 \times 1 \times 4.5$ cm quartz cuvettes were placed in a thermostated copper holder maintained at a constant temperature (±0.1 C°) and the temperatures were read by an inbuilt thermometer. The samples were continuously stirred with an inbuilt stirrer. The fluorimeter was controlled by a microprocessor, which allows averaging of any no. of measurements. However, typically 10 measurements were taken for each point. The measurement of polarization values were done by an L-format method, where the polarization (P) is given by

$$P = \frac{I_{vv} - G \cdot I_{vh}}{I_{vv} + G \cdot I_{vh}}$$

where, $I_{vv(vh)}$ is the intensity of emitted light with vertical excitation, vertical (horizontal) emission polarizer. G is the correction factor for the optical system, given by

$$G = \frac{I_{hv}}{I_{hh}}$$

$I_{hh(vh)}$ is the intensity with horizontal excitation with horizontal (vertical) emission polarizer.

DSC measurements

The DSC thermograms of DPPC was taken on Perkin-Elmer DSC-2C instrument with samples sealed in aluminium pans. The instrument was calibrated with

various standard samples covering the entire range of temperatures. The thermograms were recorded with a heating rate of 1.25 K/min and a range of 0.5 mcal/s. The lipid concentration was 60 mg/ml in phosphate buffer and fenvalerate was added to the lipid from a concentrated ethanolic stock at a final concentration of 40 μM. 15 μl of the sample was taken for each thermogram.

Results and Discussion

Fluidity of native membrane

DPH (1,6-diphenyl-1,3,5-hexatriene) is quite a common and very well known probe for monitoring the fluidity of native membrane [20–22], as well as model membranes by fluorescence polarization [21,23]. The fluorescence polarization of native membranes changes linearly with temperature which also depends on cholesterol content [2,4]. A decrease in polarization value of DPH at higher temperatures is due to marked increase in the rotational freedom of the probe upon melting of the acyl chain. The fluorescence polarization of DPH incorporated into natural membrane (synaptosomal and brain microsomal) was monitored at temperatures between 20°C and 50°C with DPH fluorescence polarization. Fluorescence spectra of DPH (2 μM) in presence of fenvalerate (40 μM) revealed absence of any quenching effect on DPH fluorescence (see methods, data not shown). Fenvalerate (20 μM) showed a considerable decrease in DPH polarization value in both synaptosomal and microsomal membrane examined (Fig. 1A and 1B). The change in DPH polarization is more at lower temperatures than at higher temperatures, which implicates, in the more ordered state of lipid the disordering effect of fenvalerate is more. The slope of the Arrhenius plot decreased with 20 μM fenvalerate compared to control and a further decrease was observed with 40 μM fenvalerate which suggests a change in the activation energy (rotational freedom) of the probe molecule. In Fig. 2 the effect of increasing concentrations of fenvalerate on DPH polarization of synaptosomal membrane at a particular temperature (37°C) is shown. At lower insecticide concentrations the change in DPH polarization is more, and at saturating concentrations the change becomes less. The nature of the curve shows that the insecticide progressively saturates the membrane at higher concentrations indicated by minimal change in fluorescence polarization value, and the curve is non-linear.

DPH is known to penetrate into the hydrophobic core and locates near the acyl chain tail portion. The change in DPH polarization observed with fenvalerate, and the non-linearity of the curve in Fig. 2 at higher concentration can be attributed to possible colocalization of fenvalerate with DPH in the hydrophobic core to quench/affect the rotational freedom of DPH by

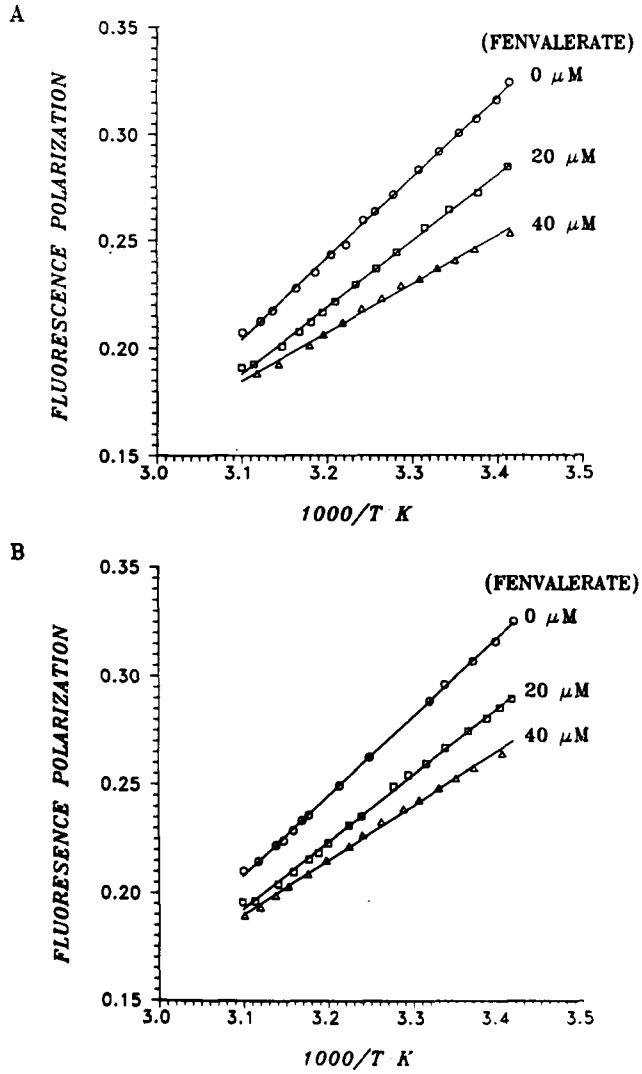

Fig. 1. Fluorescence polarization of DPH in native membranes. Synaptosomal (A) and brain microsomal (B) as a function of temperature (Arrhenius plot) in the absence (0 μM) and presence of different concentrations of fenvalerate (20, 40 μM). Best-fit lines were drawn using a linear best-fit method.

interacting with it. However, fluorescence measurements of DPH with fenvalerate in two solvents tetrahydrofuran and ethanol showed no significant change in the fluorescence spectra, ruling out the possibilities of probe quenching and direct interaction of fenvalerate with the DPH molecule. Subsequent analysis of the problem with differential scanning calorimetry measurements described below, further supports an indirect effect of fenvalerate on DPH polarization through change in membrane fluidity.

In comparison with other organochlorine insecticide effects studied so far on native membranes [2,4,5] the effect of fenvalerate is quite pronounced. For example lindane partitions into model membranes [1,24] but in in vitro experiments with isolated natural membrane it does not perturb the overall membrane fluidity [4].

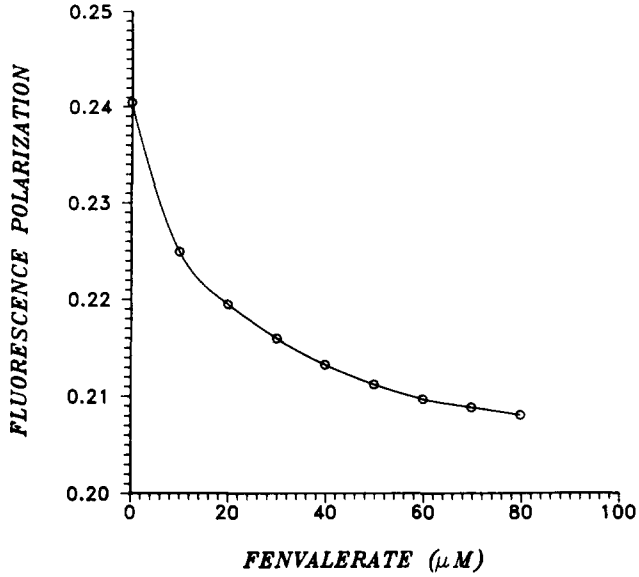

Fig. 2. Fluorescence polarization of DPH in brain synaptosomal membrane as a function of increasing fenvalerate concentration at 37°C. The best-fit curve was drawn using cubic spline best-fit method.

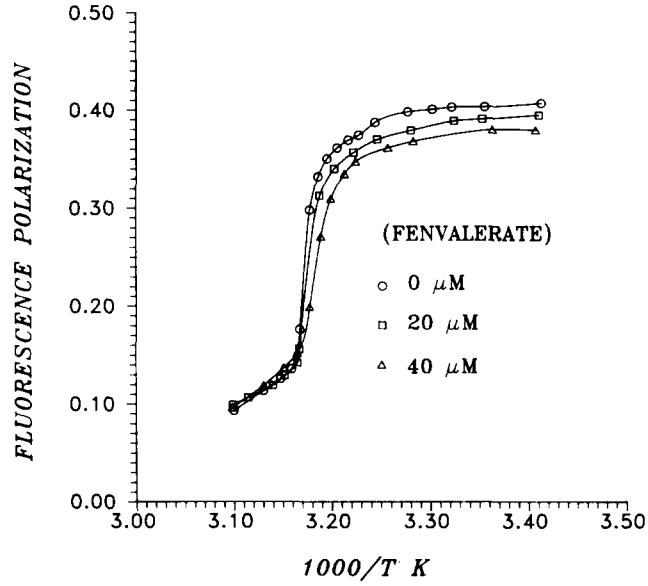

Fig. 3. Effect of fenvalerate on thermotropic phase transition property of DPPC liposomes as measured by fluorescence polarization of DPH incorporated into liposome. The midpoint of phase transition corresponds to the transition temperature (T_m). For DPPC it is 41.5°C and with 40 μM fenvalerate it changes to 40°C, determined by taking the temperature at the midpoint of steepest region of the curve.

However, systemic administration of lindane in rats changes the fluorescence polarization value of kidney brush border membranes [5]. DDT has previously been shown to lower fluorescence polarization values in native membranes (brain microsomes and erythrocytes), making it more fluid. However, unlike fenvalerate it did not change the activation energy of the probe molecule because there was no change in the slope of fluorescence polarization vs. temperature plot [2]. Fenvalerate (40 μM) reduced the fluorescence polarization value by 25% at 45°C of brain microsomal membranes in our experiments, indicating that the effect is more pronounced than the reported effect of DDT [2].

Fluidity of model membrane (DPPC liposomes)

The insecticide fenvalerate interacts considerably with DPPC bilayers (Figs. 3 and 4). The fluorescence polarization study of DPPC bilayers shows a clear decrease in transition temperature (T_m). With 40 μM of fenvalerate the change in T_m is about 1.5 C°. The decrease in T_m is also supported by DSC data (Fig. 4) as described below. The change in T_m of DPPC bilayer is consistent with the result reported previously with other organochlorine insecticides [2–4,25]. The change in T_m of DPPC liposomes with 50 μM of lindane is 1.7 C° [4] and with 50 μM of DDT, 0.4 C° [2]. Lindane does not perturb the membrane below its T_m. But, fenvalerate shows changes in membrane fluidity at temperature lower than its T_m, i.e., it also interacts with the membrane when the membrane is in gel phase like DDT. Thus fenvalerate perturbs the membrane in its gel phase and in the phase transition.

The effect of fenvalerate on the dynamic properties of model DPPC membrane was also studied by differential scanning calorimetry (DSC), which gives information about phase transition of lipid by measuring the heat capacity at different temperatures [26]. Multilamellar vesicles of DPPC gave a sharp peak for main phase transition from gel to fluid phase at 41.3°C (Fig. 4a). This change is due to the acyl chain packing of

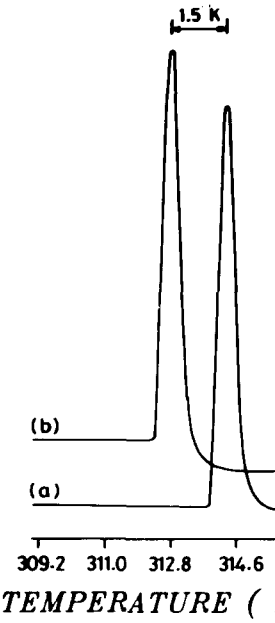

Fig. 4. DSC thermogram of DPPC (a) and DPPC with 40 μM fenvalerate (b). The Y-axis was drawn on an arbitrary scale. The peak position of control DPPC is at 41.3°C which was shifted to 39.8°C in presence of fenvalerate.

DPPC [27]. Addition of 40 μM of fenvalerate (Fig. 4b) shifts the main transition to the lower temperature by 1.5 C°, indicating that fenvalerate fluidizes the membrane, and is consistent with the result obtained from fluorescence polarization studies, though it did not show any broadening of the phase transition peak of thermogram profile. This effect of fenvalerate on DPPC shown by DSC data is very much similar to the effects of small organic solvents on lipid bilayers [28] and it is also similar to the effect of some anesthetics on membranes [29].

Jain et al. (1977) based on extensive studies on effects of different molecules on lipid bilayer by DSC, classified this kind of change in thermograms as C type change [28], where there is no broadening of phase transition peak, but T_m is shifted towards lower temperature. The characteristics of molecules which show this kind of behaviour are, they are weakly dipolar and localize in the region of the 9th or higher carbon of the acyl chain, and thereby do not modify the cooperativity of transition (sharpness of peak) but weaken the packing within the cooperative unit. In case of fenvalerate the size of the cooperative unit in the modified bilayer remains unchanged but hydrophobic tail packing of DPPC in bilayers is perturbed at lower temperature. The changes in DSC thermograms with fenvalerate suggests that fenvalerate possibly localizes itself somewhere near the C_9–C_{16} region of the bilayer. This suggestion can be supported by the fact that it also perturbs the DPH fluorescence polarization value which generally gives the averaged information about the interior environment of the bilayer. The effect of fenvalerate on fluorescence polarization of DPH incorporated in DPPC vesicles may be construed to occur in part by an inherent membrane destabilization property of DPH itself. However, an earlier study by Jain et al. [28] has confirmed that DPH per se does not affect the DSC thermogram of DPPC liposomes.

Fluidity of bilayers containing cholesterol

Fluidity of phospholipid bilayers change markedly with cholesterol [30,31]. Generally cholesterol increases the fluid phase molecular order of DMPC, DPPC bilayers, and the bilayers of DMPC or DPPC containing cholesterol are devoid of thermotropic phase transitions. Fig. 5 shows the effect of fenvalerate on DPPC bilayers enriched with cholesterol. It is evident from the plot that the disordering effect of fenvalerate is more with increase in cholesterol content of the membrane. Again like native membrane the disordering effect of fenvalerate is more in the gel phase than in the fluid phase. The effect is different from lindane [4] where it has been suggested that cholesterol might competetively inhibit the incorporation of insecticide into the membrane. But in this case the fluidizing effect of fenvalerate does not decrease with increasing

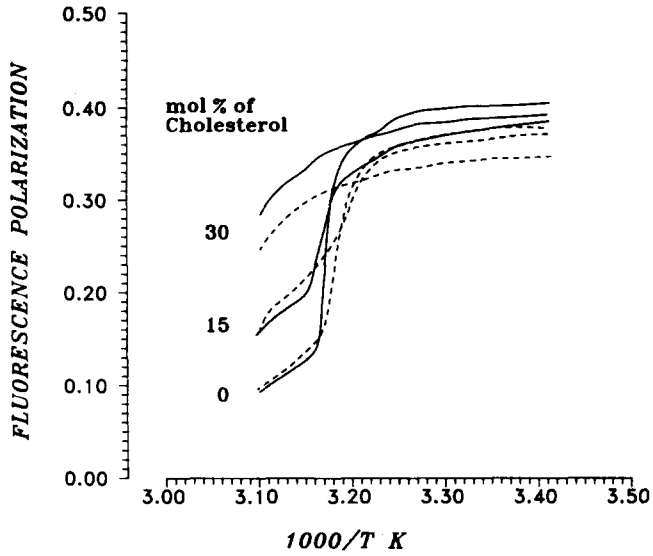

Fig. 5. Fluorescence polarization of DPH in DPPC bilayers containing cholesterol in the absence (solid lines) and in the presence (dotted lines) of 40 μM fenvalerate. Data for 0% cholesterol have been used from Fig. 3. For clarity the data points were removed from the curves.

cholesterol content, conversely it increases with increasing cholesterol content. The observation is consistent with the earlier observation of fluidizing effect of fenvalerate on native membrane where, in both the membranes studied, the extent of fluidization is more or less same with a particular insecticide concentration. This effect of fenvalerate is similar to DDT [2].

In conclusion, fenvalerate interacts with the membrane by localizing itself near the hydrophobic tail region of the lipid acyl chain and perturbs its ordered structure to make the membrane more fluid. The effect of fenvalerate on membrane ionic current specifically sodium current [10,11] might partly be exerted by this fluidization effect because change in membrane fluidity by cholesterol has previously been shown to change the ionic channel current characteristics [32], or it might directly interact with the ion channels to change their properties, which is a matter of further investigation.

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