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Organophosphorous insecticide fenitrothion alters the lipid dynamics in the spider *Polybetes pythagoricus* high density lipoproteins

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Abstract

The effect of the organophosphorous liposoluble insecticide fenitrothion (FS) on the physical properties in the lipid phase of two spider's plasma high density lipoproteins (HDL) was investigated. HDL_1 and HDL_2 have similar lipid compositions, but they significantly differ in their lipid/protein ratio and apolipoprotein compositions. Lipid dynamics in the lipoproteins' core and outer regions was determined by studying the rotational behavior of the fluorescent probes 1,6-diphenyl 1,3,5-hexatriene (DPH) and its propionic acid derivative (DPH–PA), respectively. Fluorescent steady-state anisotropy (r_s), lifetime (τ), rotational correlation time (τ_r), and the limiting anisotropy (r_∞) of these probes were measured in the lipoproteins exposed in vitro to different concentrations of FS. The results showed the FS penetration into both plasma lipoproteins, altering the lipid dynamics in their hydrophobic cores as well as in the hydrophilic outer regions. In the two lipoproteins subjected to FS action, both the limiting and steady-state anisotropy values were increased, showing an increment of the lipid ordering. The increase of r_∞ produced by FS depends on the initial lipid order, and it is higher in the less-ordered samples. Fluorescence lifetimes of DPH and DPH–PA indicate that FS increases the polarity of the probe environments, suggesting an enhanced water penetration into the lipoprotein lipid phase, possibly due to the induction of failures in the lipid packing. Its action, depending on the temperature and lipid packing in each region, affects differently either the inner or the outer portions of lipoproteins, being more affected the hydrophobic core. The alteration in the lipoprotein structure causes misfunctions in their physiological behavior, reflected in a diminished capacity of oxygen binding.

Keywords: Fenitrothion; Lipoproteins; Fluorescence anisotropy; Lipid order

1. Introduction

Organophosphorous insecticides are known to inhibit acetylcholinesterase activity, involving neuroreceptors and membrane-associated enzymes [1,2]. In this way, the toxicity associated

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with these hydrophobic insecticides was thought to be related to the composition and physicochemical properties of the biological membranes. To first address this question, several studies were carried out using as models native cellular membranes from vertebrates [3–5], invertebrates [6], and artificial membranes [7,8]. In general, certain physicochemical properties of bilayers, such as order parameters, were affected by insecticides. We then hypothesized that another lipoproteic system, as plasma lipoproteins, could be susceptible to be altered by this kind of toxin, and we recently reported that the organophosphorous insecticide fenitrothion [phosphorothioic acid, *O*,*O*-dimethyl *O*-(3-methyl-4-nitrophenyl) ester] (FS) modified differentially the physical state of the crustacean lipoproteins, depending on their lipid compositions (Garcia et al., submitted).

Insecticides are also used to control spiders, so they can be considered as targets. Knowing the way in which the insecticides are transported in the target organisms, and the relationship between the insecticide and the serum lipoproteins is important because it would determine the effectiveness of the toxic on the organism. So we now report the effect of FS on the spider *Polybetes pythagoricus* plasma lipoproteins. We used two high density lipoproteins, that we previously isolated and characterized [9,10] and they will be called HDL_1 and HDL_2. They slightly differ in their lipid compositions, but significantly differ in their lipid/protein ratio and apolipoprotein compositions. Hemocyanin is an apolipoprotein of HDL_2, so it can function as lipid as well as oxygen carrier. To assess the effect of the FS on the lipoprotein lipid moiety dynamics, the fluorescence anisotropy was measured, probing both the internal core of the lipoprotein with the fluorophore DPH and the external zones with DPH–PA. The results give information about the microviscosity of the lipoproteins in both fluorophores' environments.

2. Materials and methods

2.1. Hemolymph collection and preparative ultracentrifugal fractionation

Hemolymph from wild specimens of *Polybetes pythagoricus* was obtained as described in a previous work [9]. Plasma lipoproteins were isolated by density gradient ultracentrifugation. Aliquots of blue plasma were overlayered on NaBr solution (density 1.26 g/ml) containing 50 U/ml aprotinin (FBA Pharmaceuticals, New York) as protease inhibitor, and centrifuged at 178,000*g* for 22 h in a Beckman L8 70 M centrifuge, using a SW 60 Ti rotor. As we assumed that the plasma density was 1.006 g/ml, a saline solution of the same density was run simultaneously as blank. The total volume of the tubes was fractionated from top to bottom into 0.3 ml aliquots, and the protein content of each fraction was monitored spectrophotometrically at 280 nm.

2.2. Sample preparation and fluorescent measurement

All the measurements were made in a SLM 4800 C phase-modulation spectrofluorometer (SLM Instruments, Urbana, IL). For labeling, 3 ml of 50 mM potassium phosphate buffer, pH 7.4, with lipoproteins (0.1 mg/ml) were mixed with few microliters of concentrated dimethyl sulfoxide (DMSO) solutions of DPH or DPH–PA (final concentration 2 μM). The blanks were prepared in the same way as the samples, without the fluorescent probes, but adding the same volume of DMSO as a reference in order to correct fluorescent intensities from non-specific fluorescence and light-scattering. Samples were gently swirled at 20 °C for at least 2 h, kept in darkness out from light to allow a complete equilibration of the probes with the lipoproteins. FS from ethanolic concentrated solutions was added to samples prior to equilibration at concentrations of 1, 10, and 20 ppm, respectively.

The measurements of steady-state anisotropy (r_s) were carried out within a temperature range of 10–30 °C, though the dynamic ones were performed only at 20 and 30 °C.

2.3. Lifetime, steady-state, and dynamic polarization measurements

Steady-state anisotropy (r_s), lifetime (τ), and polarized phase shift (Δ) were measured according to Lakowicz et al. [11,12] with modifications [13,14]. Excitation wavelength was 361 nm, and the emitted light passed through a sharp cut-off filter (Schott KV 389) to eliminate the light of wavelengths below 389 nm. Measurements of τ were obtained with the exciting light amplitude-modulated at 18 and 30 MHz by a Debye–Sears modulator and vertically polarized by a Glan–Thompson polarizer. The emission light passed through the filter and then through a Glan–Thompson polarizer oriented 55 ° to the vertical to

eliminate effects of Brownian motion [15]. The phase shift and demodulation of the emitted light relative to a reference of known τ were determined and used to compute the phase lifetime (τ_p) and the modulation lifetime (τ_M) of the sample [16]. POPOP (1,4-bis(5-phenyloxazol-2-yl) benzene) in ethanol, which has a τ of 1.35 ns [12,17] was used as reference. The differential polarized phase shift (Δ) was determined according to Lakowicz et al. [11,12] by exciting with light modulated at 18 and 30 MHz and vertically polarized, and by measuring the phase difference between the parallel and perpendicular components of the emitted light. The measured values of r_s, τ, and Δ, and the fundamental anisotropy (r_0) which had been previously estimated in 0.390 [18] were used to calculate the limiting anisotropy (r_∞) and the rotation rate as previously described [13,14] in accordance with the theory developed by Weber [19].

2.4. Correlation test of FS-caused changes in limiting anisotropy

The limiting anisotropy control values from the inner (probed by DPH) and outer (probed by DPH–PA) regions of HDL_1 and HDL_2 at 20 and 30 °C were plotted versus the limiting anisotropy increment produced by the addition of 10 and 20 ppm of FS; then a minimal square linear correlation test was performed.

2.5. Detection of oxygen bound to hemocyanin

To correlate the presence of FS to HDL_2 oxygen binding, the absorption spectra of HDL_2 were analyzed in the presence and absence of FS and KCN, which inhibits the formation of the $Cu–O_2$ complex. The measurements were carried out in a SLM Aminco DW-2000 UV–Visible spectrophotometer. Samples were scanned within the wavelength range 200–400 nm, and the maximal values corresponding to protein (280 nm) and copper bound to oxygen (340 nm) were determined.

3. Results

Total protein concentrations measured in all plasma fractions after their separation by density gradient ultracentrifugation showed their maximum values in the HDL_1 and HDL_2 density regions, respectively. Both regions were isolated and used for the assays. As previously demonstrated [9,10], lipid moieties of both lipoproteins represent 30% and 3% of their total mass, respectively, and they contain 55–60% phospholipids, 15–17% triacylglycerols, 17–18% free fatty acids, and lesser amounts of diacylglycerols and sterols. The HDL_1 apolipoprotein fraction contains two polypeptides of 250 and 76 kDa, whereas the HDL_2 has hemocyanin (monomer MW 70 kDa) as the unique apolipoprotein.

3.1. FS increased steady-state fluorescence anisotropy of the lipoproteins

HDL_1 (Fig. 1) and HDL_2 (Fig. 2) were incubated with increasing FS concentrations and the fluorescent probes DPH and DPH–PA, and the steady-state anisotropy was measured. The insecticide increased the r_s values of DPH and DPH–

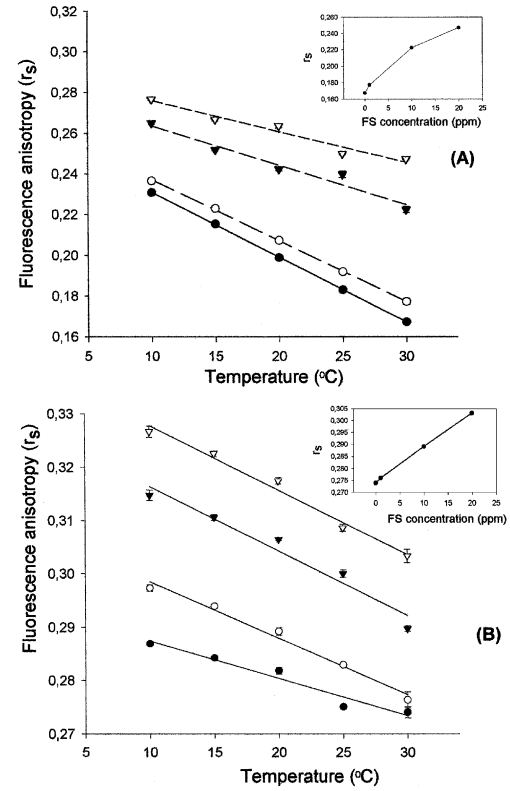

Fig. 1. Effect of FS on HDL_1. DPH (A) and DPH–PA (B) fluorescence anisotropy (r_s) versus temperature, in absence (●), presence of 1 ppm (○), 10 ppm (▼), and 20 ppm (▽) of fenitrothion. Values represent the average of five different determinations ±SD. Student's t test was used to compare the significance of the differences with respect to the sample without FS, $P < 0.0001$.

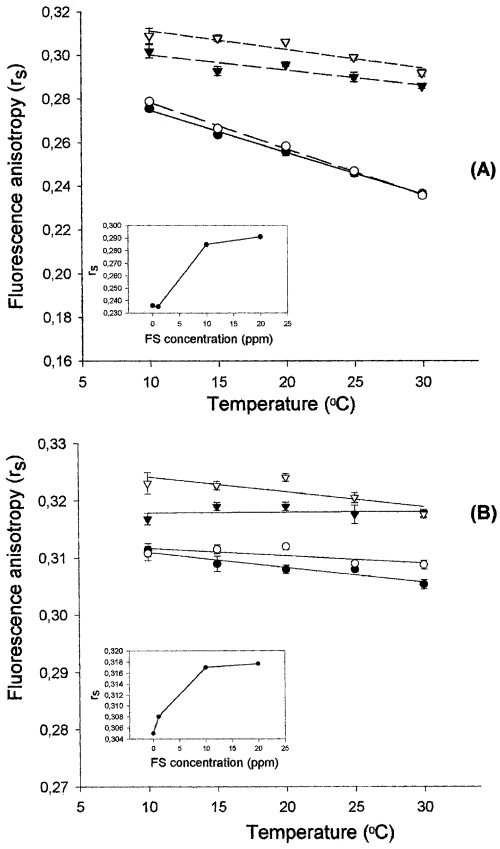

Fig. 2. Effect of FS on HDL_2. DPH (A) and DPH–PA (B) fluorescence anisotropy (r_s) versus temperature, in absence (●), presence of 1 ppm (○), 10 ppm (▼), and 20 ppm (▽) of fenitrothion. Values represent the average of five different determinations ±SD. Student's *t* test was used to compare the significance of the differences with respect to the sample without FS, $P < 0.0001$.

PA in both samples at all the temperatures tested. r_s values in all samples, including those of controls (lipoproteins not treated with FS), were higher in HDL_2 than the ones in HDL_1, consistent with the higher percentage of protein in HDL_2.

The measurements of DPH r_s on HDL_1 subjected to various FS concentrations (Fig. 1A) showed the highest increases in the anisotropy values, reaching 32–48% of control values at 20 ppm. This fact indicates that the HDL_1 core is the region more affected by the insecticide. FS significantly increased r_s, even at lower concentrations (1 ppm) in the temperature range tested. On the other hand, in HDL_1 outer regions, probed by DPH–PA (Fig. 1B), r_s values increased up to 10% of the control values, suggesting that the effect is lower when the polarity of the environment is higher.

Consistently, FS effect on HDL_2 was lower under all our experimental conditions (Fig. 2), showing a r_s increase of 20% for DPH and 4% for DPH–PA respective of the control values, when 20 ppm of FS was added. HDL_2 lacks a hydrophobic core, thus the hydrophilicity of this lipoprotein is higher and the increase of r_s was twofold higher in HDL_1, and evident only in higher FS concentrations.

FS effect on HDL_1 was proportional to its concentration in the medium (Fig. 1, insets), whereas in HDL_2 a saturation was evident with FS concentrations higher than 20 ppm (Fig. 2, insets).

3.2. Fluorescence dynamic measurements: FS decreased the fluorophores' lifetimes and increased the limiting anisotropy

Measurements of limiting anisotropy, phase and modulation lifetimes, and rotational correlation times were carried out after incorporating DPH and DPH–PA into lipoproteins, in the presence or absence of 10 and 20 ppm FS. These measurements were made at 18 and 30 MHz. Since the obtained values were similar at both frequencies, only the values at 18 MHz are shown. It is of note that calculation of τ_r and r_∞ from measurements at frequencies requires homogeneity in the rotamer fluorescence lifetime [13,14]. For both probes, but especially in the case of DPH (Figs. 3–6), modulation lifetimes (τ_M) are somewhat higher than phase lifetimes (τ_p), indicating some heterogeneity in the fluorophore population. However, the fact that the values of τ_r and r_∞ obtained are relatively independent of the frequency, indicates that they are essentially correct average values of the different rotamer populations.

Measurements of HDL_1 (Figs. 3 and 4) and HDL_2 (Figs. 5 and 6) were performed at 20 and 30 °C. Steady-state anisotropy values, already mentioned, were included in these figures for the sake of comparison. At both assayed temperatures, the fluorescence lifetimes of DPH and DPH–PA were shortened by FS in a concentration-dependent manner, suggesting an increase in the polarity of the probes' environment. The rotation correlation time of these probes was not changed by FS incorporation. However, the treatment with FS resulted in an increased limiting anisotropy for DPH at both temperatures but only at the highest assayed temperature (30 °C) for the case of DPH–PA.

To correlate the basal order of HDL_1 and HDL_2 lipids to the FS-caused increment in the limiting anisotropy, a linear correlation test was performed using the values of r_∞ obtained for both lipoproteins, using DPH and DPH–PA as fluorophores and at 20 and 30 °C. The increase in r_∞ produced by FS is inversely correlated with the basal value ($r^2 = 0.88$ for 10 ppm FS, Fig. 7A and 0.78 for 20 ppm of FS, Fig. 7B), indicating a predominant effect of FS on the less-ordered systems.

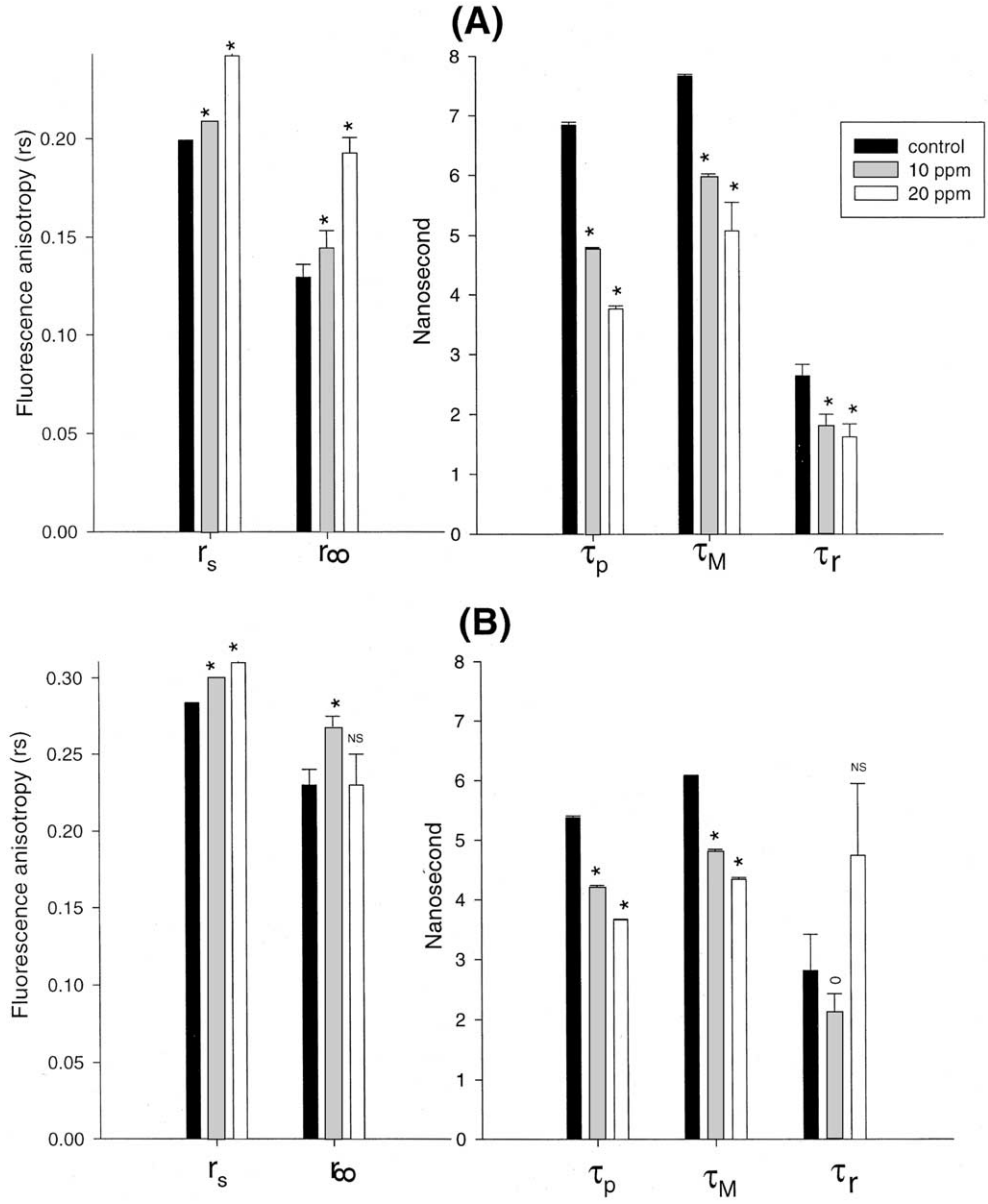

Fig. 3. Steady-state fluorescence anisotropy (r_s); phase lifetime (τ_p); modulation lifetime (τ_M); rotational correlation time (τ_r), and limiting anisotropy (r_∞) of DPH (A) and DPH–PA (B) in HDL_1 of *Polybetes pythagoricus*, measured in the absence or presence of 10 and 20 ppm of fenitrothion at 20 °C. Student's *t* test was used to compare the significance of the differences with respect to the sample without FS: $^*P < 0.0001$, $^{**}P < 0.001$, $^{***}P < 0.01$, O $P < 0.05$, OO $P < 0.06$, NS: no significant difference.

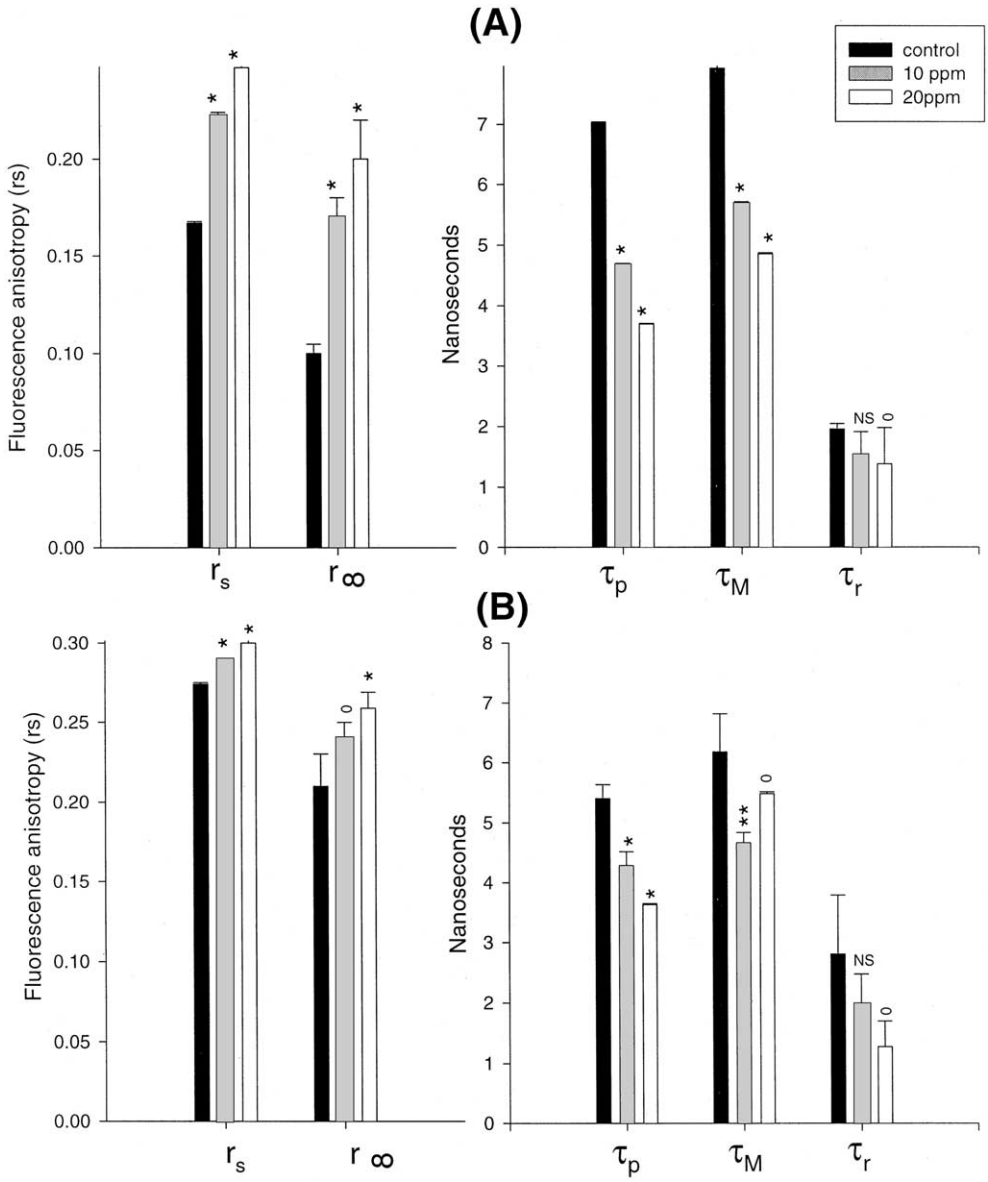

Fig. 4. Steady-state fluorescence anisotropy (r_s); phase lifetime (τ_p); modulation lifetime (τ_M); rotational correlation time (τ_r), and limiting anisotropy (r_∞) of DPH (A) and DPH–PA (B) in HDL_1 of *Polybetes pythagoricus*, measured in the absence or presence of 10 and 20 ppm of fenitrothion at 30 °C. Student's *t* test was used to compare the significance of the differences with respect to the sample without FS: $^*P < 0.0001$, $^{**}P < 0.001$, $^{***}P < 0.01$, O $P < 0.05$, OO $P < 0.06$, NS: no significant difference.

3.3. Functional implications: FS prevents the oxygenation of HDL_2

To determine whether FS alters the function of HDL_2 as an oxygen carrier, the binding of oxygen to copper was monitored spectrophotometrically at 340 nm. HDL_2 absorption spectrum was typical for an oxygenated hemocyanin, and it showed the characteristic peaks at 280 and 340 nm. In the presence of cyanide, the band at 340 nm disappears [20]. The treatment of HDL_2 with 20 ppm FS inhibited the formation of the copper–oxygen complex to an extent similar to that in the presence of 0.2 M KCN (Table 1).

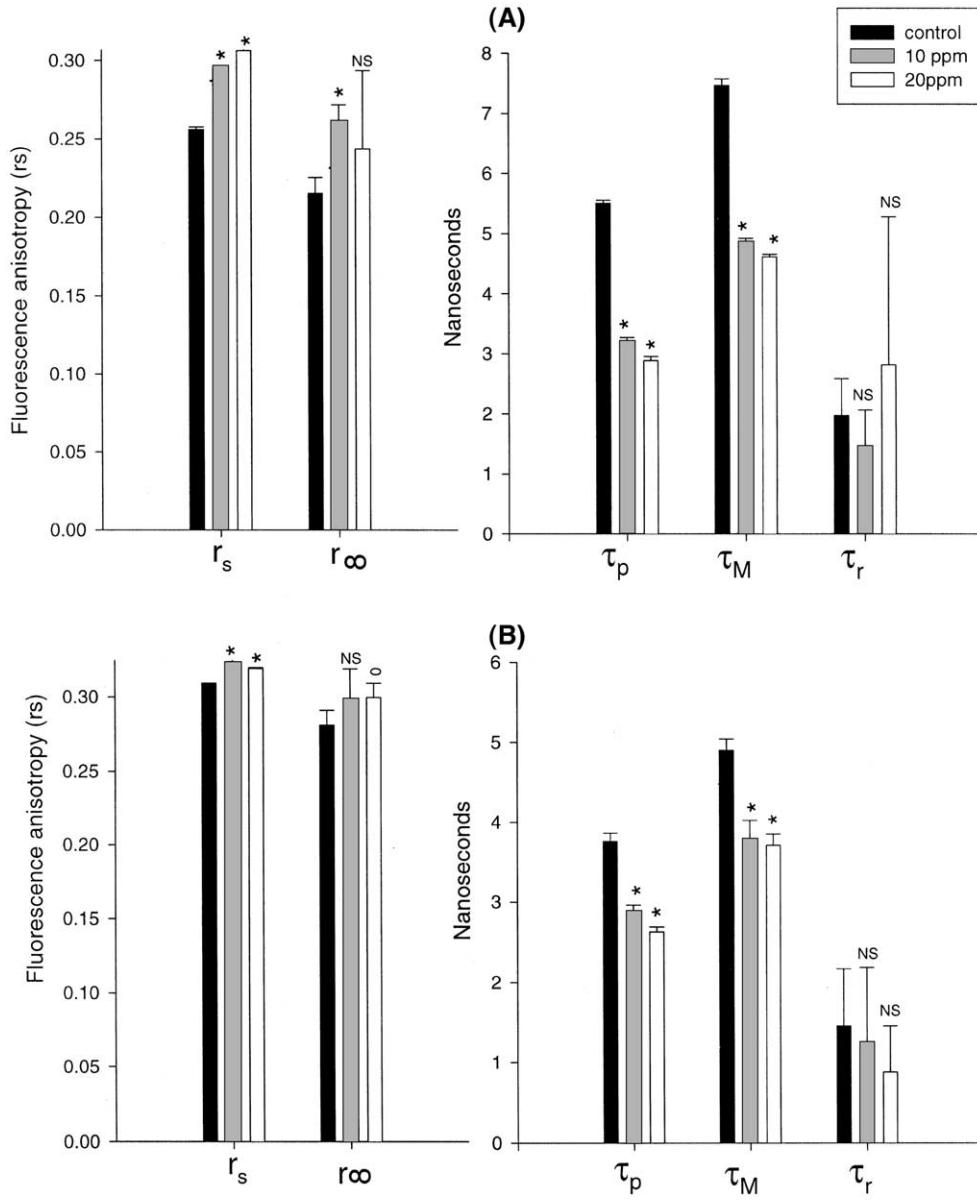

Fig. 5. Steady-state fluorescence anisotropy (r_s); phase lifetime (τ_p); modulation lifetime (τ_M); rotational correlation time (τ_r), and limiting anisotropy (r_∞) of DPH (A) and DPH–PA (B) in HDL_2 of *Polybetes pythagoricus*, measured in the absence or presence of 10 and 20 ppm of fenitrothion at 20 °C. Student's *t* test was used to compare the significance of the differences with respect to the sample without FS: $*P < 0.0001$, $**P < 0.001$, $***P < 0.01$, O $P < 0.05$, OO $P < 0.06$, NS: no significant difference.

4. Discussion

The effect of fenitrothion upon the lipid dynamics of two spider hemolymphatic lipoproteins was studied by fluorescence techniques. These lipoproteins have a similar proportion of lipid classes, but their lipid/protein ratios, however, are entirely different. Lipid packing in different regions of the lipoprotein structure was measured using two fluorescent probes. One of them, DPH, is an elongated molecule that is a useful fluores-

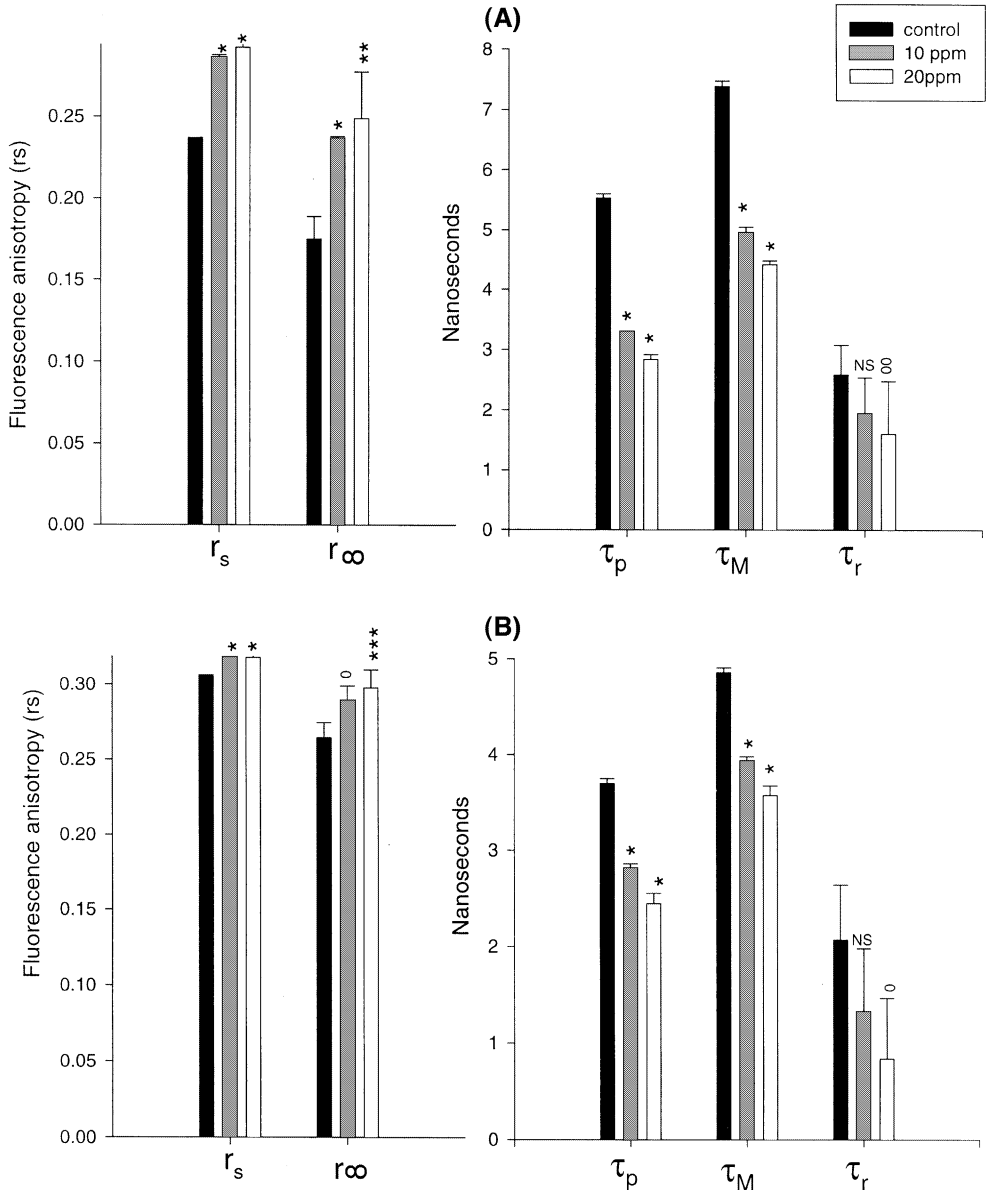

Fig. 6. Steady-state fluorescence anisotropy (r_s); phase lifetime (τ_p); modulation lifetime (τ_M); rotational correlation time (τ_r), and limiting anisotropy (r_∞) of DPH (A) and DPH–PA (B) in HDL_2 of *Polybetes pythagoricus*, measured in the absence or presence of 10 and 20 ppm FS at 30 °C. Student's *t* test was used to compare the significance of the differences with respect to the sample without FS: $*P < 0.0001$, $**P < 0.001$, $***P < 0.01$, O $P < 0.05$, OO $P < 0.06$, NS: no significant difference.

cent probe for studying the alterations in the lipid packing order in the inner part of a membrane or lipoprotein. It is known to penetrate deeply into the hydrophobic zone, and to locate in the acyl chain portion. The carboxylate group of DPH–PA interacts with the polar headgroups of the phospholipids, fixing the fluorescent moiety at a short distance from the polar/non-polar interface. Both probes are preferentially oriented with their long axis parallel to the acyl chains, and the motion that causes depolarization is the wobbling displacing this long axis.

The values of the steady-state fluorescence anisotropy (r_s) evidenced, in both lipoproteins, an

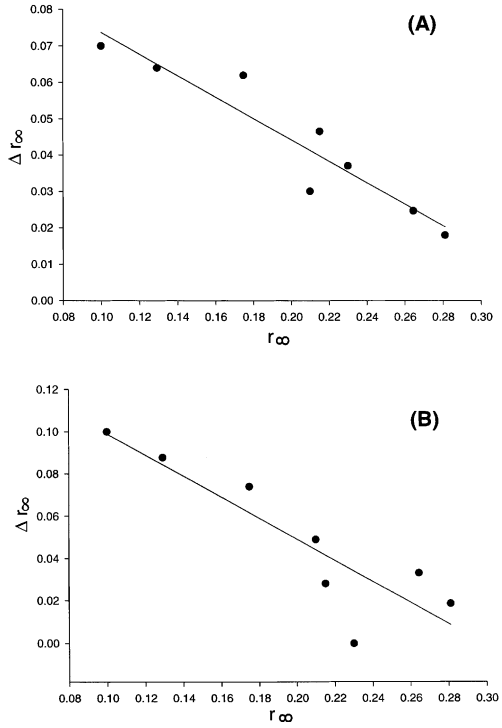

Fig. 7. Linear correlation between the control r_∞ of both lipoproteins and Δr_∞. The r_∞ values from HDL_1 and HDL_2 obtained using DPH and DPH–PA as fluorophores at 20 and 30 °C were plotted. Δr_∞ was calculated as: $(\Delta r_\infty = r_\infty$ (with FS) $- r_\infty$ (without FS)). (A) FS, 10 ppm and (B) 20 ppm.

increment proportional to the FS concentration in the incubation medium. This result indicates that the insecticide penetrates into their lipid moieties altering their properties. Since this alteration in the physical state can be observed with both fluorophores, it may be inferred that FS alters not only the hydrophobic internal region, but also the region near the polar/non-polar interface in both lipoproteins. We have also demonstrated that FS has a rigidizing effect when inserted into lipid membranes [6]. Using a similar method, we have studied the effect of FS on two circulating lipoproteins of crustacean, having different lipid composition but the same total lipid/total protein ratio (Garcia et al., submitted). On these crustacean lipoproteins, FS have also a rigidizing effect but some quantitative differences were found which were attributed to the different lipid composition. In the present work, in which two lipoproteins of similar lipid composition are compared, the higher lipid packing found for HDL_2 even in the absence of FS should be ascribed to other characteristics such as the low lipid/protein ratio. Although only the lipid moiety of both lipoproteins was tested, the influence of an apoprotein of high molecular weight such as hemocyanin (usually present as an hexamer, dihexamer, or heptamer [10,21]) on the HDL_2, cannot be ruled out. The HDL_1, due to its lipid and apoprotein composition, might have an external phospholipid monolayer and an internal core of neutral lipids similarly to the insect lipophorins. In the HDL_2, the small amounts of lipids can be bound to hydrophobic regions in the hemocyanin, without forming a true lipid monolayer nor a hydrophobic core. So the different response of HDL_1 and HDL_2 r_s to increasing concentrations of FS in the medium may be due to a higher availability of lipids in HDL_1 than HDL_2.

Table 1
FS inhibits the oxygen binding to hemocyanin

HDL_2	+	+	+
20 ppm FS	–	+	–
0.2 M KCN	–	–	+
$OD_{340/280\,nm}$	0,230	0,170	0,172

The Cu–O_2 complex formation in hemocyanin was determined by measuring the absorbance at 340 nm. The values were normalized for the protein concentration and expressed as OD_{340}/OD_{280}. Three-hundred micrograms of HDL_2 protein were incubated with either 20 ppm FS or 0.2 M KCN.

In both lipoproteins subjected to FS action, the limiting anisotropy values were found to be increased, thus corroborating the increment of the lipid ordering.

It must be pointed out that the increase of r_∞ produced by FS depends on the initial lipid order and is higher in the less-ordered samples. This fact might be due to a preferential partition of FS in the less-ordered samples. However since FS decrease the fluorescence lifetime of DPH or DPH–PA in a similar extension in both lipoproteins, a different partition of FS should be discussed. Moreover, we have previously shown [8] that although FS partitions similarly in liquid-crystalline or gel state lipid bilayers, it increases extensively the order in liquid-crystalline state but not in the highly ordered gel state.

The decline of the τ_p and τ_M values of DPH and DPH–PA would be the consequence of failures in the lipid packing, produced by FS action, which would increase the amount of water into the lipid phase of the lipoproteins. It is very unlikely that the decrease in lifetime is the result of

the quenching of the fluorophore fluorescence produced by FS, since it has been previously reported that intensity and lifetime of DPH fluorescence in ethanol were not affected by a large excess of FS [6]. This effect of FS, reported here, has been shown repeatedly in different studies using microsomal membranes [6], phosphatidylcholine liposomes [8], or crustacean lipoproteins (Garcia et al., submitted), and it seems to be a constant feature which differentiates FS from other insecticides. This is the case of pyrethroids, which showed shorter lifetimes of the fluorophore and a decrease in the lipid order when assayed on phospholipid liposomes [22].

In brief, it was assessed that FS is able to penetrate into both hemolymphatic lipoproteins of spiders, altering them differently when compared to other insecticides or other disturbing agents. It increases the lipid order in a proportional fashion to concentration in HDL_1, and increases water penetration into both lipoproteins' lipid phases. Its action, depending on the temperature and lipid packing in each region, affects differently either the inner or the outer portions of lipoproteins. The oxygen-carrier capacity of the FS-treated HDL_2 was also altered. The formation of the copper–oxygen complex was inhibited in a similar way to that of cyanide, thus decreasing the respiratory efficiency of hemocyanin. So, we can conclude that structural changes in the lipoproteins evoked by FS may affect their functionality.

Acknowledgments

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