Effect of Fenitrothion on the Physical Properties of Crustacean Lipoproteins

C.F. Garcia, M. Cunningham, M.R. González-Baró, H. Garda*, and R. Pollero

Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), Consèjo Nacional de Investigaciones Científicas y Técnicas (CONICET)-Universidad Nacional de La Plata (UNLP), (1900) La Plata, Argentina

ABSTRACT: The effect of the liposoluble organophosphorus insecticide fenitrothion (FS) on lipid packing and rotation of two crustacean plasma HDL was investigated. These lipoproteins, HDL-1 and HDL-2, differed in their lipid composition, but their lipid/protein ratios were similar. The rotational behavior of the fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid (DPH-PA) was used to obtain information about the lipid dynamics in the outer and inner regions, respectively, of the lipid phase of the lipoproteins. 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 to different concentrations of FS *in vitro*. The results showed the penetration of FS into both plasma lipoproteins, altering the lipid dynamics of the inner as well as the outer regions. The overall effect of the insecticide was to induce an increase in the lipid order in a concentration-dependent fashion. DPH and DPH-PA fluorescence-lifetime shortening indicated that FS increased the polarity of the probe environment, suggesting an enhanced water penetration into the lipoprotein lipid phase, may be due to the induction of failures in the lipid packing. Even in the absence of FS, a higher ordering of the lipid phase was found in HDL-2 compared to HDL-1, a fact that might be attributed to a higher percentage of sphingomyelin in HDL-2.

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The toxic effects of organophosphorus insecticides on target and nontarget organisms have been described in several studies, although only a few have reported the effects of these xenobiotics on the lipid metabolism. These insecticides are known to cause neurotoxic effects by inhibiting acetylcholinesterase and other enzymes (1,2). The proper function of integral membrane proteins depends on the environment, specifically, the membrane structure. This fact prompted several groups to investigate the alterations in the physicochemical properties of membranes produced when these insecticides, which are mostly liposoluble, are inserted into the lipid bilayers. In this regard, studies done in natural membranes from mammals and some other vertebrates (3–5), an invertebrate (6), and in artificial membranes (7,8) have shown the overall alterations on membrane lipid dynamics produced by organophosphorous insecticides.

We hypothesized that these liposoluble xenobiotics might also alter other lipoprotein systems such as the circulating lipoproteins, so we carried out the present work using as models the insecticide fenitrothion (FS) and two high-density hemolymphatic lipoproteins from the decapod crustacean *Macrobrachium borellii*. Lipids in crustaceans are transported by HDL-1 and HDL-2 (9). HDL-1 is present in the plasma of both males and females. In *M. borellii* it is a particle of 295 kDa composed of three apolipoproteins of 124, 26, and 23 kDa that transport exogenous and endogenous lipids. HDL-2, also called vitellogenin, is only present in females during the vitellogenic periods; its function is to carry the yolk precursors from extraovarian synthesis sites to the ovocyte. The *M. borellii* native lipoprotein (440 kDa) has three apolipoprotein subunits (94, 26, and 23 kDa) (Garcia, C.F., unpublished results). Although both serum lipoproteins have high phospholipid contents, their lipid compositions are different. Taking into account the different functions and phospholipid contents of HDL-1 and HDL-2, and their similar lipid/protein ratio, we considered them as an appropriate model to study the effect of insecticides on the physicochemical properties of lipoproteins, correlating their structural and functional characteristics with FS sensitivity. This may be of physiological interest since alterations in the physical properties of these lipoproteins affect lipid transport *in vitro*, and they may influence the lipid exchange among tissues.

The rotational behavior of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid (DPH-PA) was studied to investigate the effect of FS on the dynamics of the lipoprotein lipid phase. Whereas the neutral DPH senses mainly the hydrophobic deep interior of the lipoprotein lipid phase, the anionic DPH-PA is anchored to the interface through its carboxylate group, and it locates its fluorescent moiety more externally. Fluorescence lifetime, and steady-state and frequency-resolved anisotropy measurements were made. They allowed us to evaluate the polarity of the probe's environments, as well as to resolve the probe rotation in terms of rate and amplitude, which are indicative of the lipid phase fluidity and ordering, respectively.

MATERIALS AND METHODS

Sampling and isolation of lipoproteins. Male and ovogenic female adult specimens of *M. borellii* were collected in a

*To whom correspondence should be addressed at INIBIOLP, Fac. de Cs. Médicas, UNLP, Calles 60 y 120, La Plata (1900), Argentina.
E-mail: hagarda@isis.unlp.edu.ar

Abbreviations: Δ, polarized phase shift; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPH-PA, 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid; FPLC, fast-flow protein liquid chromatography; FS, fenitrothion; r_0, fundamental anisotropy; r_s, steady-state anisotropy; r_∞, limiting anisotropy; τ, lifetime; τ_M, modulation lifetime; τ_P, phase lifetime; τ_r, rotational correlation time.

watercourse close to the Rio de la Plata, Argentina. After severing their heads, the shrimps were placed in a tube and centrifuged at low speed to obtain hemolymph. The lipoproteins under study were isolated by density gradient ultracentrifugation. Aliquots of plasma were overlayered on 3 mL NaBr solution (density 1.26 g/mL) containing 0.01% sodium azide and centrifuged at 178,000 × *g* at 10°C for 24 h in a Beckman L8 70-M centrifuge, using a SW60 Ti rotor. The total volume of the tubes was fractionated from top to bottom into 0.2-mL aliquots. The protein content of each fraction was monitored spectrophotometrically at 280 nm. One tube containing a NaCl solution (density 1.04 g/mL) instead of plasma was centrifuged simultaneously and fractionated in order to determine the density of the fractions by monitoring the refraction indexes.

Lipid and protein analysis. Lipids were extracted following the method of Folch *et al.* (10). Quantitative determinations of lipid classes were performed by TLC coupled to an FID in an Iatroscan apparatus Model TH-10, after separation on Chromarods SIII, using a triple development solvent system as described previously (11). All lipid classes were quantified using MAG as internal standard. Total protein concentration in each fraction isolated from the density gradient was measured colorimetrically by the method of Lowry *et al.* (12).

In vitro *determination of* FA *binding to HDL*-1. Total serum of *M. borellii* was incubated with of 0, 20, and 40 ppm FS for 2 h. Afterward, the FA-binding capacity was assayed with 0.5 μCi (9 nmol) of [1-^{14}C]palmitic acid (NEN, Boston, MA) as ammonium salt for 30 min. To isolate the labeled HDL-1, plasma hemolymph was analyzed under nondenaturing conditions by preparative gel filtration fast-flow protein liquid chromatography (FPLC) on a Superdex 200 HR 10/30 column (Amersham- Pharmacia, Uppsala, Sweden) using 0.1 M Tris-HCl pH 8.0 at a flow rate of 0.4 mL/min. Protein was detected at 280 nm. The column was calibrated with thyroglobulin, ferritin, BSA, and ribonuclease A (Amersham-Pharmacia) as protein markers. The HDL fraction was collected based on the retention time and relative mass ratio. The identity of HDL-1 was corroborated by PAGE, the protein concentration was determined (12), and the radioactivity of the palmitic acid bound to each protein peak was quantified by liquid scintillation counting.

Fluorescent measurement. All measurements were made in a SLM 4800 C phase-modulation spectrofluorometer (SLM Instruments Inc., Urbana, IL). For labeling, 3 mL of buffer solution lipoproteins (100 μg/mL) were mixed with a few microliters of concentrated DMSO solutions of DPH or DPH-PA (final concentration 4 μM). Blanks were prepared in the same way as samples, without the fluorescent probes but with addition of the same volume of DMSO as reference in order to correct fluorescence intensities for nonspecific fluorescence and light-scattering. Samples were gently swirled at 20°C for at least 2 h, in the absence of light, to allow a complete equilibration of the probes with the lipoproteins. FS from ethanolic concentrated solutions (1, 10, and 20 ppm) was added to samples prior to equilibration.

Lifetime, steady-state, and dynamic polarization measurements. Polarized phase shift (Δ), steady-state anisotropy (r_s), and lifetime (τ) were measured according to Lakowicz *et al.* (13,14) with modifications (15,16). The excitation wavelength was 361 nm, and the emitted light passed through a sharp cut-off filter at 389 nm to eliminate 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 (17). 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 (17). POPOP (1,4-bis(5-phenyloxazol-2-yl)benzene) in ethanol, which has a τ of 1.35 ns (14–19) was used as reference. The differential polarized phase shift (Δ) was determined according to Lakowicz *et al.* (13,14) 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 previously been estimated as 0.390 (20), were used to calculate the limiting anisotropy (r_∞) and the rotational correlation time (τ_r), which is the inverse of the rotational rate, as previously described (15,16) in accordance with the theory developed by Weber (21).

RESULTS

Separation and analysis of lipoproteins. Plasma hemolymph was separated by density gradient ultracentrifugation into HDL-1 (hydrated density 1.13 g/mL), which is found in animals of both sexes, and HDL-2 (hydrated density 1.18 g/mL), which is found only in females at the vitellogenic stage.

Lipid composition, determined by TLC-FID, is shown in Table 1. The predominant lipid class in both lipoproteins is PC, which is found in a higher proportion in HDL-1, whereas

TABLE 1
Lipid Composition[a] and Lipid/Protein Ratio of Lipoproteins Isolated from Plasma of *Macrobrachium borellii*

	Fraction	
Lipid classes	HDL-1	HDL-2
TAG (%)	15.2 ± 2.3	16.3 ± 0.8
FFA (%)	11.7 ± 0.6	12.3 ± 0.7
Cholesterol (%)	14.6 ± 2.0	12.3 ± 2.0
PE (%)	19.5 ± 0.3	16.9 ± 1.9
PC (%)	38.8 ± 4.5	22.1 ± 2.3
Sphingomyelin (%)	Trace	18.8 ±1.7
Total lipids (mg/mL hemolymph)	3.3 ± 0.4	4.67 ± 0.4
Total proteins (mg/mL hemolymph)	2.9 ± 0.3	4.45 ± 0.2
Lipid/protein ratio	1.13	1.04
Hydrated density (g/mL)	1.11–1.13	1.16–1.19

[a]Data on lipid classes are expressed as weight percentage as determined and quantified by TLC-FID. Values represent the mean ± SD of three independent analyses.

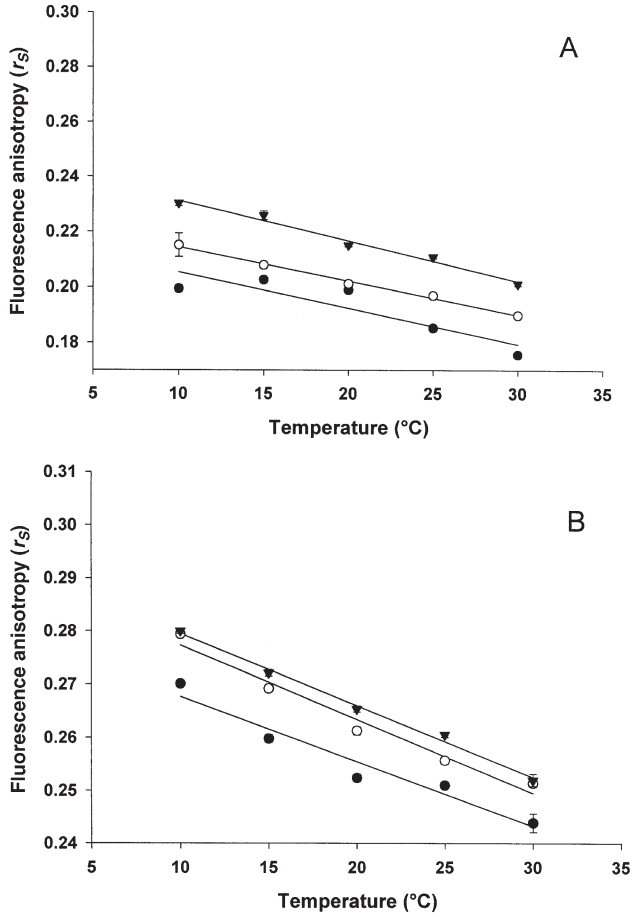

FIG. 1. Effect of fenitrothion (FS) on HDL-1. 1,6-Diphenyl-1,3,5-hexatriene (DPH) (A) and 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid (DPH-PA) (B) fluorescence anisotropy (r_s) vs. temperature, in the absence (●) and the presence of 10 (○) and 20 (▼) ppm of FS. Values represent the average of five different determinations ± SD. The linear correlation coefficients ranged between 0.790 and 0.999.

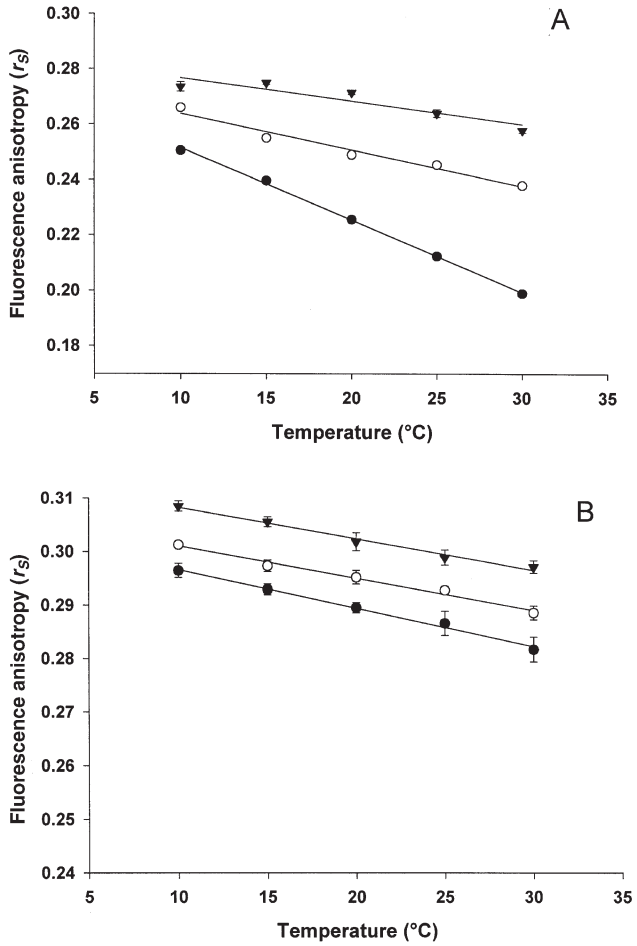

FIG. 2. Effect of FS on HDL-2. DPH (A) and DPH-PA (B) fluorescence anisotropy (r_s) vs. temperature, in the absence (●) and the presence of 10 (○) and 20 (▼) ppm of FS. Values represent the average of five different determinations ± SD. The linear correlation coefficients ranged between 0.850 and 0.999. For abbreviations see Figure 1.

the percentages of PE, TAG, cholesterol, and FFA did not show significant differences between HDL-1 and HDL-2. A relatively high content of sphingomyelin was evident in HDL-2, but it was not present in HDL-1.

The lipid/protein ratio (lower in HDL-2) was consistent with the hydration densities (higher in HDL-2).

FS increases steady-state anisotropy of DPH-derived fluorophores in HDL-1 and HDL-2. Different concentrations (1–20 ppm) of FS affect the microenvironment of the fluorescent probes in HDL-1 (Fig. 1) and HDL-2 (Fig. 2). FS increases r_s either in the hydrophobic core of the lipoproteins, probed with DPH (Fig. 2A), or in the outer regions of the lipoproteins, probed with DPH-PA (Fig. 2B). The observed effect is dependent on the concentration of the pesticide, and it is constant within the temperature range 10–30°C. We used that temperature range because it represents the extremes of temperature recorded in the stream where these animals live.

Effect of FS on the lifetimes of DPH and DPH-PA and on the lipid order of HDL-1 and HDL-2. To better understand the effect of FS on the rotational behavior of DPH and DPH-PA, lifetime and differential polarized phase shifts (Δ) were measured. They were used to calculate τ_r and r_∞ in HDL-1 (Fig. 3) and HDL-2 (Fig. 4). These measurements were made at 18 and 30 MHz. Since the values obtained at both frequencies were similar, only the values of 18 MHz are shown. It is notable that calculation of τ_r and r_∞ from measurements at discrete frequencies requires homogeneity in the rotamer fluorescence lifetime (15,16). For both probes, but especially for DPH (Figs. 3A and 4A), 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.

A significant shortening was observed in the fluorescence lifetime (τ_P and τ_M) of DPH and DPH-PA incorporated into HDL-1 (Fig. 3) or HDL-2 (Fig. 4) in which different concentrations of FS were added at 10 or 30°C. In the case of HDL-1, FS incorporation did not alter the rotational correlation time (τ_r) of DPH or DPH-PA (Fig. 3). FS also evoked a concentration-

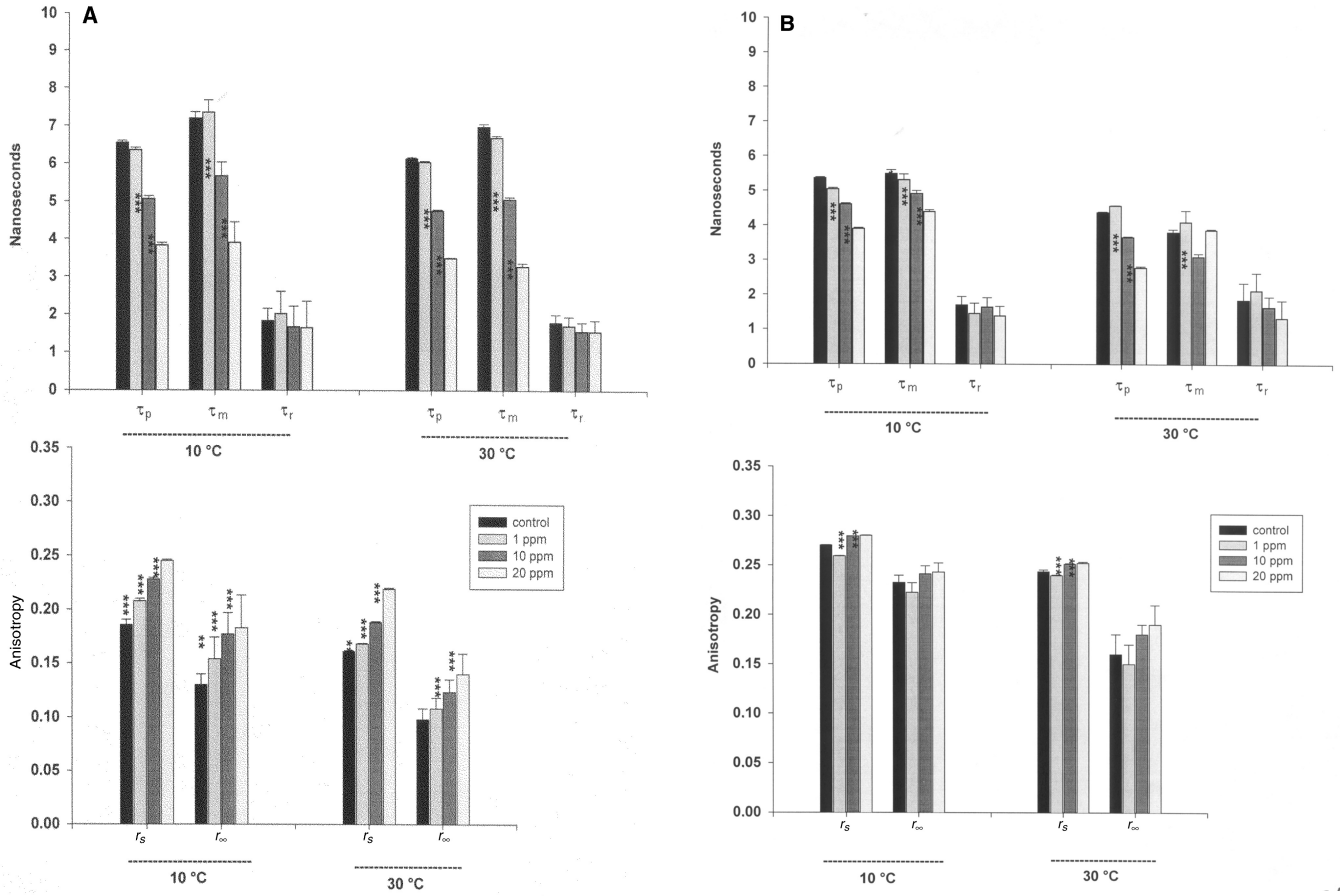

FIG. 3. Steady-state fluorescence anisotropy (r_s), phase lifetime (τ_P), modulation lifetime (τ_M), rotational correlation time (τ_r), and limiting anisotropy (r_∞) of (A) DPH and (B) DPH-PA in HDL-1 of *Macrobrachium borellii,* measured in the absence or presence of 1, 10, and 20 ppm FS at 10 and 30°C. Student's *t*-test was used to compare the significance of the differences with respect to the sample without FS: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

dependent increase in the steady-state and limiting anisotropy of both probes in HDL-1, at both temperatures.

Figure 4(A and B) shows the effect of FS incorporation on HDL-2. A decrease of τ_r for DPH after the addition of the toxin was found at both temperatures, whereas for DPH-PA, it was observed only at 30°C. As was found for HDL-1, FS increased r_∞ significantly for both probes in HDL-2. These results indicate that FS incorporation generated a strong effect on the lipid phase dynamics of HDL-1 and HDL-2 at both temperatures.

From comparison of both lipoproteins (Figs. 3 and 4), it is evident that for both probes, higher r_s and r_∞ values were obtained in HDL-2 than in HDL-1. This fact indicates that HDL-2 has a more ordered lipid phase than HDL-1. On the other hand, the fluorescence lifetimes and τ_r of both probes were similar in both lipoproteins.

FS decreases palmitic acid binding to HDL-1. In the presence of ^{14}C-labeled palmitic acid, the only radioactive fraction detected in nonvitellogenic *M. borellii* hemolymph was the one corresponding to the HDL-1. The FPLC retention time and PAGE of the eluted fractions corroborated its identity. When exposed to FS, palmitic acid bound to HDL-1 decreased 1.6-(20 ppm FS) and 12.6-fold (40 ppm FS), i.e., the radioactivity was 64 and 9% compared to the control, respectively.

DISCUSSION

Several organophosphorus, chlorinated, and pyrethroid insecticides were reported to affect the fluidity of native and model membranes (22–34). In previous studies using the same insecticide, we demonstrated on the basis of fluorescent anisotropy measurements that FS increases the lipid order in the bilayer of *M. borellii* microsomal membranes, but it does not alter the rotational rate of the DPH and DPH-PA (6). In the present study, the spectroscopic properties of two fluorescent probes located in different regions of the lipoprotein were used to investigate the influence of the insecticide FS, at different concentrations, on the physical state of the lipid phase of two *M. borellii* lipoproteins. One of the probes, DPH, is an elongated molecule, which is a useful fluorophore for studying alterations in the lipid packing and ordering. It penetrates the hydrophobic core of membranes and locates in the acyl chain region. The carboxylate group of the other fluorophore, DPH-PA, interacts with the polar headgroups of the phospholipids,

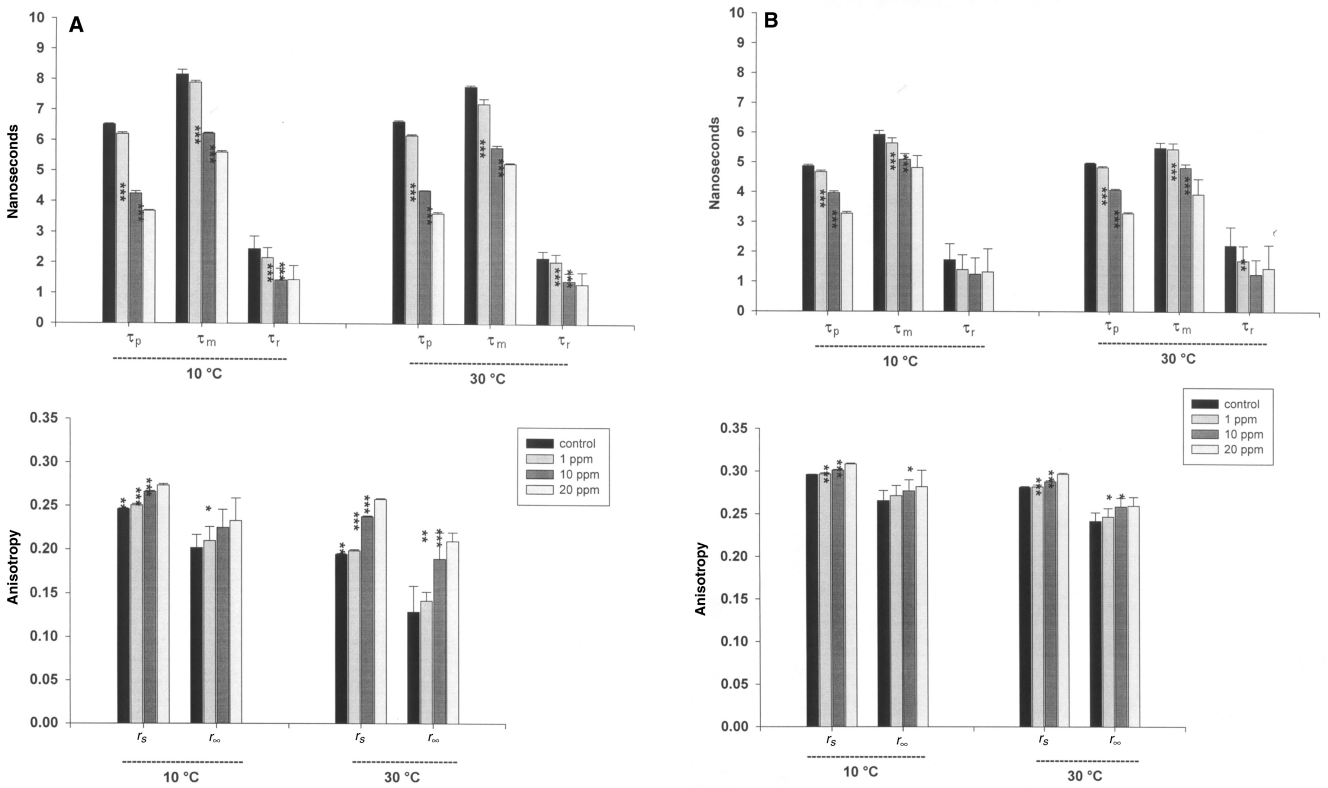

FIG. 4. Steady-state fluorescence anisotropy (r_s), phase lifetime (τ_P), modulation lifetime (τ_M), rotational correlation time (τ_r), and limiting anisotropy (r_∞) of (A) DPH and (B) DPH-PA in HDL-2 of *M. borellii*, measured in the absence or presence of 1, 10, and 20 ppm FS at 10, and 30°C. Student's *t*-test was used to compare the significance of the differences with respect to the sample without FS: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

locating the fluorescent moiety more outwardly than in the case of DPH. Both probes are preferentially oriented with their long axis parallel to the acyl chains, and the motion that causes depolarization is the wobbling that displaces this long axis.

The increase in r_s of DPH and DPH-PA generated by FS addition demonstrated that this insecticide modifies the physical properties of the lipid phases in the hydrophobic core as well as in the outer region of both lipoproteins. A rigidifying effect of FS is suggested by the increased r_s values, although this general parameter depends not only on the rotational behavior but also on the fluorescent lifetime. The shortening in the fluorescent lifetime produced by FS is one of the facts causing the increase in r_s. However, the results of the differential polarization phase measurements confirm that FS produces a rigidifying effect on these lipoproteins by increasing the lipid phase ordering, evoked through the hindering of the probes' wobbling amplitude resulting in an increased r_∞. On the other hand, the rotational rate of these probes is not restricted by the incorporation of FS. At least at 30°C, FS seems to increase the rotational rate of both fluorophores slightly, as indicated by the decrease in τ_r, in HDL-2, but τ_r remained unchanged in HDL-1 after FS addition. We can therefore assume that the main parameter modified by FS is the lipid order.

The interaction of pyrethroids with liposomal phospholipids evidenced a lower lipid order and a shortening in the DPH lifetime (23). On the contrary, the present results showed that FS decreased DPH and DPH-PA fluorescence lifetime but increased the lipid order in HDL-1 and HDL-2. It is unlikely that this lifetime decrease is due to the direct quenching by FS of the probes' fluorescence, since as previously demonstrated the fluorescence intensity and lifetime of DPH in ethanol were not affected by a large excess of FS (6). Then, the observed decrease in the fluorescence lifetime of these probes is caused by the alterations produced by FS within the structure of the lipoprotein lipid phase. Defects caused by FS in the lipid packing can allow increased water penetration into the hydrophobic interior of these systems, consistent with observations in natural (6) and artificial membranes of 1-palmitoyl-2-oleoyl and dipalmitoyl PC (8).

The action of organophosphorus insecticides on membranes has been related to cholesterol content in those membranes (5). Thus, high proportions of cholesterol preclude the insecticide insertion into the membrane. Although cholesterol content is relatively high in both lipoproteins of *M. borellii*, this does not seem entirely to hinder FS insertion into the lipid phase.

The present results also indicate that HDL-2 has a higher ordering of the lipid phase than HDL-1, a fact that can be at-

tributed to the different lipid compositions of both lipoproteins. The main difference in composition is the presence of sphingomyelin in HDL-2 but not in HDL-1. It is well-known that sphingomyelin has a rigidifying effect on membranes (35), lipid monolayers (36), liposomes (37), and some human lipoproteins (38), but its physiological function in plasma is still controversial. It was postulated that sphingomyelin may act as an inhibitor of lipoprotein peroxidation because of its rigidifying effect, which may hinder the lateral propagation of the lipid-peroxy radicals (39). In this way, the presence of sphingomyelin in HDL-2 rather than in HDL-1 could be important for yolk synthesis. DNA damage in the embryo could be minimized if the precursor is peroxide-poor. This fact would explain the higher r_s and r_∞ values found in HDL-2. Thus, FS is able to penetrate the lipid phase of HDL-2 and produce a further ordering increase; this effect is especially noticeable at 30°C.

In brief, these results show that FS can be a perturbing agent to the circulating lipoproteins of *M. borellii*, increasing their lipid phase order, producing packing defects, and thus affecting their structures and functions. In HDL-1, a decrease in the binding of palmitic acid was observed, implying that the proper structure of the lipoprotein is essential for the lipid-binding capacity. Although PC is the major lipid class in HDL-1, we have previously observed in *in vivo* studies that HDL-1 can exchange FA with the hepatopancreas in *M. borellii* (40). In this regard, insecticide actions can be important from both the physiological and toxicological points of view.

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