Lipid thermotropic transitions in *Triatoma infestans* lipophorin

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Abstract   The structure and lipid thermotropic transitions of highly purified lipophorin of *Triatoma infestans* were examined by several techniques: steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), *cis*-parinaric acid (*cis*-PnA) and *trans*-parinaric acid (*trans*-PnA), light scattering fluorescence energy transfer between the lipophorin tryptophan residues and the bound chromophores, DPH, *trans*-parinaric acid *cis*-parinaric acid, gel electrophoresis, and gel filtration. Fluorescence polarization of PnAs and DPH revealed a reversible lipid thermotropic transition in intact lipophorin at about 20°C and 18°C, respectively. In lipophorin, lipid dispersion fluorescence polarization of DPH detected a lipid transition approximately at 20°C, while *trans*-PnA showed a gel phase formation at a temperature below 30°C. Similar experiments in which *trans*-PnA was incorporated into diacylglycerols and phospholipids extracted from the lipophorin revealed gel phase formation below 30°C and 24°C, respectively. Light scattering measurements showed that lipophorin particles aggregate irreversibly at 45°C, increasing the molecular weight, as determined by gel filtration on Sephacryl S-300, from 740,000 to values larger than 1,500,000. The particle aggregation did not change the physical properties of the lipophorin studied by fluorescence polarization, indicating that the aggregation is apparently a non-denaturing process. Energy transfer between the lipophorin tryptophans and the bound chromophores *cis*-PnA, *trans*-PnA, and DPA revealed a different location of the fluorescent probes within the lipophorin. Temperature-dependence on the energy transfer efficiency for all probes confirmed a change in the ordering of the lipophorin lipids at 24°C — **Soulages, J. L., O. J. Rimoldi, and R. R. Brenner.** Lipid thermotropic transitions in *Triatoma infestans* lipophorin. *J. Lipid Res.* 1988. **29:** 172–182.

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Ultracentrifugation of *Triatoma infestans* hemolymph yields three lipoprotein fractions: a high density lipoprotein (HDL) and two very high density lipoproteins. HDL is largely the principal carrier of lipid, transporting diacylglycerols from storage sites, but also from absorption sites, to other tissues (1, 2). HDL corresponds to the lipoprotein designated lipophorin by Chino et al. (3). Diacylglycerols, phospholipids, and hydrocarbons are the principal components of lipophorin (1).

The metabolism of insect lipophorins may be a function of their composition, structure, and physical properties, which can be altered under different physiological conditions and temperature.

It has been shown that, in locust, the lipoprotein diacylglycerols and molecular weight are elevated several-fold in response to adipokinetic hormone release (4–7).

Phospholipids of locust lipophorin are on the surface of the particle (8) and hydrocarbons appear to occur as a nonpolar domain within the lipophorin having two endothermic peaks centered at 11°C and 36°C (9).

Studies of lipophorin structure are at an early stage, and no information about the problems of protein–lipid interactions and of the state and organization of lipids in the presence or absence of proteins has been reported yet.

Fluorescent probes have been very useful to elucidate structural and dynamic properties of human or animal lipoprotein lipids (10–13). Schroeder, Goh, and Heimberg (14) and Sklar et al. (15) have shown that the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) is located in the interior core of human lipoproteins, while *trans*-parinaric acid (*trans*-PnA) is exclusively located on the surface of the lipoprotein particles. Moreover, Sklar, Miljanich, and Dratz (16) showed that *trans*-PnA, due to its high solubility and quantum yield in gel phases, is a useful fluorescent probe to detect low proportions of this phase, while *cis*-PnA has the same solubility in both gel or liquid crystalline phases. The fluorescence polarization of these three probes embedded into the lipophorin may give valuable information on different structural aspects and temperature-dependent changes of the lipophorin lipids. Moreover, resonance energy transfer from the apoprotein tryptophan residue to these three fluorescent probes can

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Abbreviations: HDL, high density lipoproteins; DPH, 1,6-diphenyl-1,3,5-hexatriene; *cis*-PnA, *cis*-parinaric acid; *trans*-PnA, *trans*-parinaric acid; DG, diacylglycerol; PL, phospholipid.
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give information about the relative localization of the apoproteins in the surface or core of the lipoprotein particle (10, 14, 17).

In the present report, temperature-dependence on the fluorescence polarization of DPH, *trans*-PnA and *cis*-PnA, energy transfer experiments, and 90° light scattering were employed to examine structure and lipid thermotropic transitions in the 5°–60°C temperature range. The term lipid thermotropic transition is used in a general sense to denote a thermally induced change in the physical state of the lipophorin lipids. The change might involve order–disorder transitions of the liquid crystalline to gel type, lateral phase separations, or lipid clusters (18).

MATERIALS AND METHODS

Insects

Adult female *T. infestans*, reared on hen blood twice a week and maintained at 28°C and 60–70% humidity in our laboratory, were used. Seven or 8 days after the last moult, the insects were fed and then fasted for 5 days prior to the collection of the hemolymph.

Collection of hemolymph

Insects were anesthesized by ice cooling. To collect hemolymph, the legs were cut off and the insect was placed in a precooled centrifuge tube with the head towards the pointed end and centrifuged for 1 min at 120 g and 4°C. Hemolymph was then centrifuged at 4°C and 10,000 g for 10 min to remove hemocytes. Darkening of hemolymph by air exposure was prevented by addition of 5 mmol/ml hemolymph of N-acetyl-L-cysteine.

Isolation of lipophorin

Hemolymph was ultracentrifuged to fractionate the lipoproteins, using a procedure similar to that described by Fichera and Brenner (1). Three ml of NaBr solution (density 1.28 g/ml) was placed in a centrifuge tube and 1 ml of hemolymph was carefully layered over the solution and centrifuged at 178,850 g at 10°C for 20 hr. The lipophorin band was removed by aspiration. For purification of lipophorin, this fraction was mixed with NaCl solution (d 1.006 g/ml) to a total volume of 1 ml and layered over 3 ml of NaBr solution (density 1.18 g/ml) and centrifuged at 178,800 g at 10°C for 10 hr. Lipophorin was dialyzed at 4°C for 24 hr against 50 mM potassium phosphate buffer, pH 7.2, 130 mM NaCl, and 1 mM EDTA.

Determination of the lipophorin size

The Stokes radius was estimated by gel permeation on Sephacryl S-300 column (80 × 1.6 cm) and eluted with the dialysis buffer at a flow rate of 20 ml/hr at room temperature. Fractions of 2 ml were collected and peak frac-

tions were detected by absorbance at 280 nm. High molecular weight calibration standards (Pharmacia Fine Chemicals) were used to estimate molecular weight, and void volume was measured with Blue Dextran (Pharmacia). Stokes radius (R_s) was estimated by gel filtration using a calibration curve in which R_s was plotted against $(-\log K_{av})^{1/2}$. The partition coefficient K_{av} was calculated according to

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_e is the elution volume, V_0 and V_t are void and total volumes, respectively.

Gel electrophoresis

Non-dissociating polyacrylamide slab-gel electrophoresis was performed in 5% resolving gel with 1 cm of 3.75% stacking gel following the method of Frings, Foster, and Cohen (19). The gel was stained with Coomassie Blue G-250.

Lipid extraction and separation

Total lipids were extracted from the lipoproteins by the method of Folch, Lees, and Sloane Stanley (20). Diacylglycerols were separated by thin-layer chromatography (TLC) on silica gel G plates developed in hexane–diethylether–acetic acid 80:20:2 (v/v) as solvent mixture. Appropriate standards were visualized by exposure to iodine vapor. Unexposed diacylglycerol zones were scraped off the plate and extracted with chloroform–methanol–hexane 2:1:3 (v/v). The total lipid extract (in chloroform) was chromatographed on a column packed with silicic acid (Mallinckrodt 100 mesh). Neutral lipids were eluted with chloroform and phospholipids were eluted with methanol.

Spectroscopic measurements

An Aminco-Bowman spectrofluorometer was used for the fluorescence and 90° light-scattering experiments. For the polarization measurements, the spectrofluorometer was equipped with two Glan Thompson polarizers. The polarization alignment was verified using fluorescein in alkaline glycerol at 10°C (21). The polarization ratio P is defined as $I_{\parallel}/I_{\perp} \cdot G$. $I_{\parallel}$ and $I_{\perp}$ refer to intensities emitted, parallel and perpendicular, respectively, to the vertically polarized excitation, and G is a factor for instrument correction (22). The maximum reached by P in this system was 2.75 ± 0.05. Excitation wavelengths of 360, 315, and 320 nm, and emission wavelengths of 430, 420, and 410 nm were used for fluorescence polarization of DPH, *trans*-PnA, and *cis*-PnA, respectively. A 2.0-M NaNO$_2$ solution placed between the emission monochromator and the photomultiplier was used as a cut-off filter for the wavelengths below 390 nm. Light scattering

was less than 5% and 15% in the fluorescence polarization of DPH and PnA, respectively. Unlabeled samples with the same concentration of the labeled samples were used as reference blanks which served to correct for the excitant light-scattering and other contributions to the fluorescence signal.

The steady-state fluorescence anisotropy (r_s) of DPH was calculated using the equation:

$$r_s = \frac{P - 1}{P + 2}$$

where P is the polarization ratio previously defined.

Temperature was monitored by a thermocouple inserted directly into the cuvette.

Flourescence quenching and uncorrected emission spectra were measured in 10×10 mm cuvettes. Corrections by the inner filter-effect never exceeded 10% of the fluorescence intensity. Tryptophan excitation was performed at 280 nm to reduce direct excitation of the fluorescent probes.

Preparation of lipophorin and lipid dispersion for the fluorescence measurements

Lipophorin (250 μg of protein/ml) suspended in 50 mM potassium phosphate buffer, pH 7.2, was labeled by adding 3–5 μl of trans-PnA or cis-PnA (PL-Biochemicals) in ethanolic solution, or DPH (Aldrich Chemical Co.) in tetrahydrofurane. The concentration of the parinaric acids in stock solution was determined by measuring absorbance using the extinction molar coefficients of Sklar et al. (23), and DPH concentration was determined in hexane according to Shinitzky and Barenholz (24). The particle concentration was calculated from the protein content determined by the method of Lowry et al. (25) in the presence of SDS using bovine serum albumin standard. Lipid samples were dried under N_2 in the tubes and then sonicated in 50 mM potassium phosphate buffer, pH 7.2, at 40–45°C, for 10 min in a Heat System sonicator. In both lipid dispersion or lipophorin suspensions, the molar ratio of DPH to lipids was about 1:250, and the PnA to lipid molar ratio was about 1:100.

Binding studies

The binding of trans-parinaric acid and cis-parinaric acid was studied by fluorescence enhancement, according to the method described by Sklar (26). The partition constant K_p defined as:

$$K_p = \frac{\text{moles of bound PnA/moles lipophorin superficial lipids}}{\text{moles of free PnA/moles of } H_2O}$$

was obtained from the slope of the double reciprocal plot

of I^{-1} against moles of H_2O/moles of superficial lipids (SL). It is based on the equation:

$$\frac{1}{I} = \frac{1}{I_{max}} + \frac{1}{K_p \, I_{max}} \cdot \frac{\text{mol } H_2O}{\text{mol SL}}$$

where I_{max} is the maximal fluorescence intensity of PnA calculated from the y intercept. The moles of superficial lipids were calculated from the weight percent of the phospholipids, cholesterol, and diacylglycerols of the lipophorin (1), using the following molecular weights: DG = 626; PL = 780; and cholesterol = 386.

Calculation of Arrhenius plots

To determine the best fits and the breakpoints of the Arrhenius plot, data obtained in the fluorescence anisotropy studies were processed as follows.

A linear plot was computed for the first three data points and a second line was computed for the remainder of the data points by least squares fit. The variance of the data points around the two lines was computed. This was repeated for the first four points, five points, etc., until all possible two-line fits were computed. The fit with the minimum variance was chosen as the best two-line fit. To decide whether the best two-line fit was significantly better than the best one-line fit (least square fit of all the data points), an F test was performed to approximate the statistical significance of the difference in variance between one-line fit and the two-line fit. No two-line fit was accepted unless the F test indicated statistical significance ($P < 0.05$).

Other methods

Phospholipids were determined by the method of Chen, Toribara, and Huber (27); total lipids were determined gravimetrically; and diacylglycerols were determined by gas–liquid chromatography in a Hewlett Packard model 5840-A, calculating the content of fatty acid methyl esters employing 1,2-dieicosaenoylglycerol as an internal standard.

RESULTS

Lipophorin isolation, purity, and size

As shown in **Fig. 1**, native gel electrophoresis of our lipophorin preparation demonstrates a single major band with small amounts as aggregated material that can be attributed to the so-called "O" lipophorin (28). After Sephacryl S-300 gel filtration, lipophorin emerged as a single symmetrical peak, and no protein peaks were eluted either before or after the lipophorin peak. The lipophorin preparation was homogeneous in size and no significant contamination with either larger or smaller
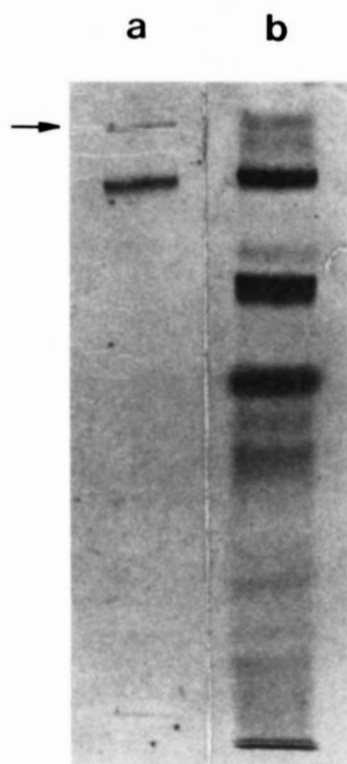
Fig. 1. Native polyacrylamide gel electrophoresis of lipophorin after second density gradient ultracentrifugation (lane a), and whole hemolymph (lane b). Analyses were in 5% resolving gel with 3.75% of stacking gel. The arrow indicates the origin of separating gel.

proteins could be detected. A value of 73 Å was obtained for the *T. infestans* lipophorin Stokes radius. It indicates that *T. infestans* lipophorin size is in the range of the other insect lipophorins (29).

Fluorescence polarization studies

Within several limitations (30), the fluorescence anisotropy is inversely related to the relative motional freedom of the fluorophore in the lipids and provides thereby an indication of the "fluidity" of lipid molecules in the lipoprotein particles. In order to determine the contribution of the different lipid classes and apoproteins to the overall lipid dynamics in intact lipophorins, and to obtain a better understanding of the thermotropic behavior of the lipophorin lipids, the temperature-dependence on the fluorescence polarization of DPH, *trans*-PnA, and *cis*-PnA was studied in purified intact lipophorin. In addition, the total lipophorin lipids and the two major lipidic components of the lipophorin (1), diacylglycerols and phospholipids, were examined by fluorescence polarization of DPH and *trans*-PnA. Arrhenius plots of the fluorescence anisotropy of DPH are shown in **Fig. 2**, and **Table 1** summarizes the values of the breakpoints observed in the lipophorin and lipophorin components. As determined by linear regression analysis, the data for lipoprotein, total

lipids, and total diacylglycerols fit best in a two-slope plot. A breakpoint at around 18°C was found in whole lipophorin, while total lipophorin lipids and diacylglycerols showed higher breakpoints at 20°C and 26°C, respectively. Fig. 2 shows heating curves. However, cooling curves of lipophorin produced similar breakpoints and no significant hysteresis was detected.

Lipophorin hydrocarbons comprise a 10% (w/w) of the *T. infestans* lipophorin lipids (1). Hydrocarbons can be incorporated into the lipophorin by incubation of an aqueous lipophorin suspension with the extracted and purified hydrocarbon fraction. Incorporation of an additional 10% of hydrocarbons into the native lipophorin evoked only a slight increase of the fluorescence anisotropy of DPH and a 3°C increase of the breakpoint temperature (data not shown).

Fluorescence anisotropy values show that phospholipids are the most fluid components of the lipophorin, and the comparison between the r_s values of the total lipid dispersion and the intact lipophorin (Fig. 2 and Table 1), indi-
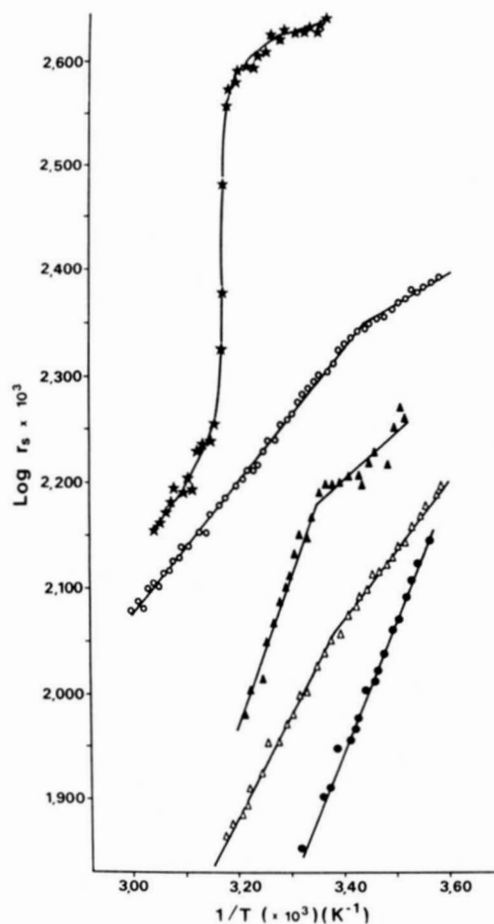
Fig. 2. Arrhenius plots of steady-state anisotropy of fluorescence of DPH in DPPC (★), intact lipophorin (○), total lipophorin lipid dispersion (△), total lipophorin diacylglycerol dispersion (▲), and total lipophorin phospholipid dispersion (●).

TABLE 1. Fluorescence anisotropy (r_s) of DPH at 25°C and breakpoints (t_o) of lipophorin and lipophorin lipid fractions

Fraction	t_o	r_s
Lipophorin	18	0.200
Lipids	20	0.108
Diacylglycerides	26	0.157
Phospholipids		0.078

cate that apoproteins produce an elevated restriction on the mobility of the lipids.

The data on the fluorescence polarization of the amphipathic fluorescent probes cis-PnA and trans-PnA (**Fig. 3**) show that in the native lipophorin there is a small phase separation that begins at about 20°C, while in the total lipophorin–lipid dispersion labeled with trans-PnA, a gel phase formation begins at about 30°C. In addition, diacylglycerols and phospholipid fractions showed slope changes at 30°C and 25°C, respectively, which indicate that a fraction of these lipid classes forms a gel phase. Similar to what was found with DPH, the higher values of the fluorescence polarization of trans-PnA found in native lipophorin compared to values for the total lipophorin lipids indicate an elevated ordering effect of the apoproteins over the lipophorin lipids.

Fluorescence spectra of lipophorin

Fluorescence is a classical technique to study the environments of aromatic residues in proteins (31). This technique has also been used to examine the interactions between apoproteins and lipids (32) and between enzymes and lipids (33, 34).

Triatoma infestans lipophorin contains two apoproteins; the major one, apoLpI (mol wt 255,000) contains about 90% of the total lipophorin–tryptophan content (Rimoldi, O. J., personal communication). Thus, the intrinsic fluorescence spectrum of lipophorin is dominated by the apoLpI fluorescence spectra. When apoLpI is unfolded in the presence of 6 M guanidine-hydrochloride, it exhibits a maximum in the fluorescent spectrum at 351 nm (**Fig. 4**), which is a typical value of tryptophan completely exposed to water (31). On the other hand, typical values of fluorescence spectra maximum for tryptophan in nonpolar environments are in the range of 330–332 nm. In our experiment the intrinsic fluorescence spectrum of lipophorin in 50 mM potassium phosphate buffer, pH 7.2, exhibited a maximum at 339 nm ± 1. Therefore, this value would correspond to a tryptophan in limited contact with water, probably immobilized by bonding at the macromolecular surface. In consequence, these results suggest that the apoprotein tryptophans in intact lipoproteins are partially exposed to water.

Energy transfer studies

The absorption spectra of cis-PnA, trans-PnA, and DPH overlap the emission spectrum of lipophorin. This feature

permits the use of the fluorescence energy transfer to these ligands to measure distance relationships (35–37). For lipoproteins containing several tryptophan residues whose quantum yields and spatial locations are not known, energy transfer measurements cannot give unequivocal information on interchromophoric distances. However, the choice of trans-PnA and cis-PnA as fluorescence acceptors, which have similar spectral overlapping of tryptophan (38) and different gel–liquid crystalline partition coefficients (16), makes them suitable for study of the heterogeneity and nature of the lipophorin–lipid domains.

The effects of the incorporation of cis-PnA over the lipophorin–tryptophan fluorescence are illustrated in **Fig. 5**. The spectra show the decrease in the intensity of the lipophorin–tryptophan fluorescence upon addition of cis-PnA to the lipophorin particles at 6°C. The peak intensities from these spectra are plotted in **Fig. 6** as quenching curves. In addition, the data for trans-PnA and DPH, also at 6°C, are presented in Fig. 6. Results show that the quenching produced by cis-PnA is slightly more efficient than the quenching evoked by trans-PnA, while DPH evokes the lowest quenching effect. Since the spectral overlap of the DPH–tryptophan pair is larger than that of the PnA–tryptophan pairs it would be possible to expect a larger quenching effect in the lipophorins labeled with DPH. Therefore, the observation of larger quenching in the lipoproteins labeled with PnA than with DPH clearly indicated a larger average distance between the DPH–tryptophan pairs than between the PnA–tryptophan pairs.
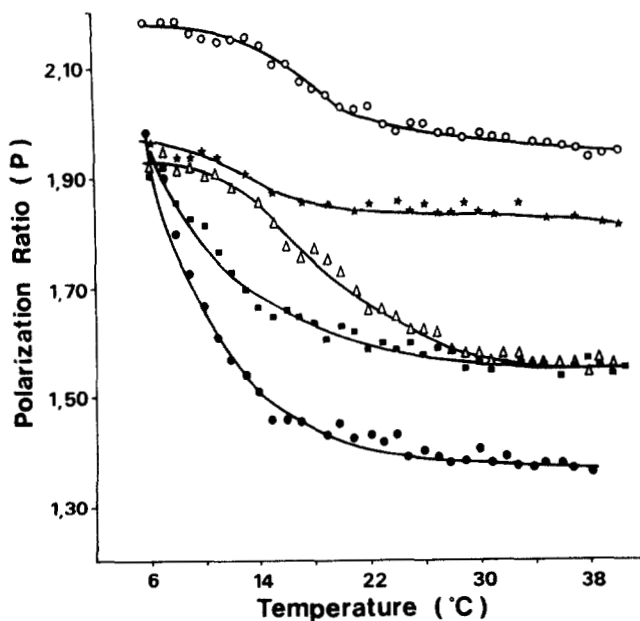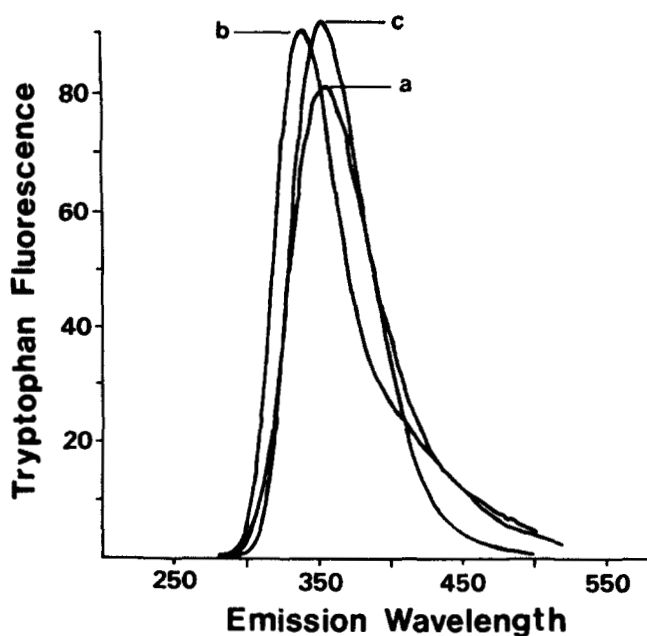

Fig. 3. Temperature-dependence on the polarization of fluorescence of cis-PnA in intact lipophorin (★), and trans-PnA in intact lipophorin (○), total lipophorin lipid dispersion (■), total lipophorin diacylglycerol dispersion (△), and total phospholipid dispersion (●).

Fig. 4. Fluorescence spectra of: a) apoLp$_{\text{I}}$, 50 μg of protein/ml in 6 M guanidine hydrochloride; b) lipophorin, 200 μg of protein/ml in potassium phosphate buffer, pH 7.2; c) tryptophan, 60 μM, in potassium phosphate buffer, pH 7.2.

Approximate temperature-dependence of lipophorin fluorescence quenching was also recorded. **Fig. 7** shows that a temperature increase from 24° to 30°C evokes a sharp decrease in the energy transfer efficiency for all the probes used and the values at lower temperatures are in good agreement with those of Fig. 6.

One possible reason for the change in the PnA quenching efficiency could be due to a change in the amount of the polyene fatty acid chromophore in the lipophorin. Decreased quenching efficiency of parinaric acids above 24°C could be due to a decreased association of the probes with the lipophorin. This possibility was examined by determining the binding association constants of polyene fatty acid probes at 6°C and 37°C. **Fig. 8a** shows the binding titration curves obtained at 6°C and Fig. 8b shows the double reciprocal plots of the data of Fig. 8a. At 6°C, K_p values of 7.2×10^6 and 2.9×10^6 were found for *cis*-PnA and *trans*-PnA, respectively, whereas K_p values of 5.6×10^6 and 3.3×10^6 were found at 37°C for *cis*-PnA and *trans*-PnA, respectively. Thus, this factor cannot evoke a variation of the tryptophan fluorescence intensity, since more than 98% of the added parinaric acids was bound at any temperature in the working conditions of the experiments.

Light scattering studies

Light scattering techniques can render information about the lipid thermotropic transitions in membranes and lipoproteins (39, 40) and can also give information about the particle size or particle size changes. **Fig. 9** shows the temperature-dependence on the 90° light scattering of lipophorin suspension studied in the 5°–60°C temperature range. The heating curve shows that between 22° and 44°C there is a sharp decrease in the light scattering intensity, while above 44°C the light scattering intensity increases at least up to 62°C. The curves also show that when the lipophorin suspension was heated to 32°C, the cooling curve gave results similar to the heating curves, indicating a reversible process. However, when the lipophorin suspension was heated up to 60°C and then cooled, a different curve was obtained, indicating that above 44°C an irreversible process is produced. The decrease in the light scattering intensity that begins at 22°C is a typical change observed in order–disorder transitions, while the increase in the light intensity observed above 44°C can be explained by the size increase of the particles. The size increase was further investigated by heating a lipophorin suspension at 50°C for 20 min and applying the sample in a chromatography gel-filtration column packed with Sephacryl S-300. After this process no lipophorin peak was detected and all the sample eluted in the void volume, indicating that, in our working conditions and at temperatures higher than 44°C, the lipophorin particles aggregate irreversibly to form particles with molecular weights larger than 1,500.
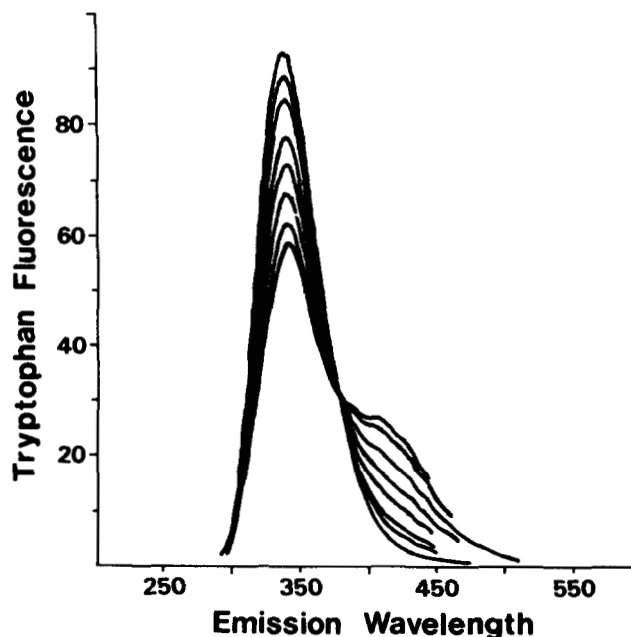
Fig. 5. Quenching curves. Fluorescence emission spectra of lipophorin particles were recorded as described in Material and Methods. Aliquots of a stock ethanolic solution of 0.22 mM *cis*-PnA were added to 2 ml of lipophorin resuspended at a final concentration of 300 μg of protein/ml. Sample temperature was 6°C.
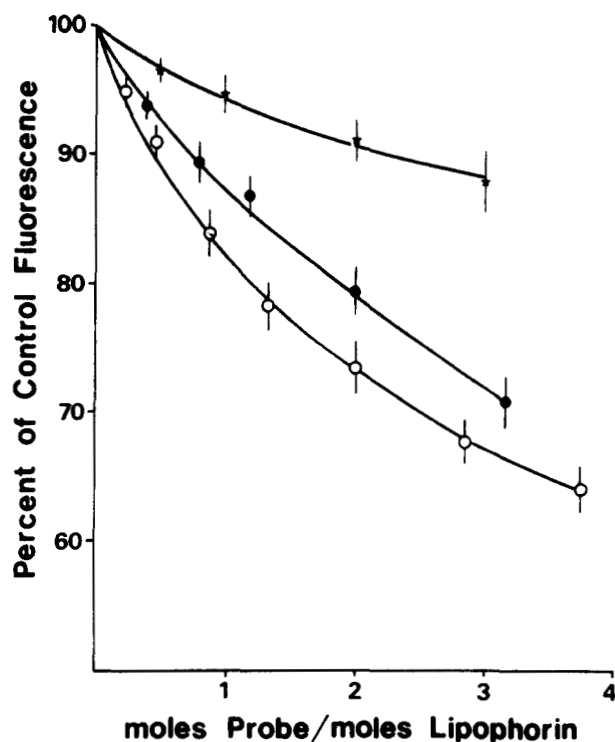
Fig. 6. Decrease in lipophorin tryptophan fluorescence quenching by *cis*-PnA (O), *trans*-PnA (●), and DPH (★). Percent of control fluorescence was determined from emission curves as described in the legend to Fig. 5. Lipophorin:probe molar ratios were calculated using a lipophorin molecular weight value of 740,000. Results are the mean of three determinations ± SD.

DISCUSSION

T. infestans lipophorin is a lipid-transporting lipoprotein with a density between 1.11 and 1.15 g/ml (1). The lipid content is high, 30 to 50%, but with a completely different composition compared to vertebrate lipoproteins (41). Unlike lipoproteins from vertebrates it contains high amounts of diacylglycerols (29.2%) and hydrocarbons (10.0%), and low amounts of triacylglycerols (6.6%). It also contains phospholipids (31.4%), cholesterol (9.4%), and cholesteryl esters (3.9%). The two main phospholipids are phosphatidylethanolamine and phosphatidylcholine. But phosphatidylethanolamine (67.6%) predominates over phosphatidylcholine (32.4%). The fatty acid composition of lipophorin lipids has been already reported (1, 41) and shows the predominance of fatty acids of 16 and 18 carbons, mainly oleic, palmitic, and stearic acids. Linoleic acid is also present in a fairly constant proportion of about 7%.

At least two types of thermally induced structural changes take place in the *Triatoma infestans* lipophorin within the 5°–60°C temperature range: *a*) a reversible lipid thermotropic transition in intact lipophorin particles and *b*) an irreversible lipoprotein particle aggregation.

The reversible lipid thermotrophic transition in native lipophorin that falls at about 20°C and 18°C has been shown by fluorescence polarization of PnAs and DPH, respectively. Correspondingly a lipid transition at about 20°C has been detected in the total lipophorin lipid dis-
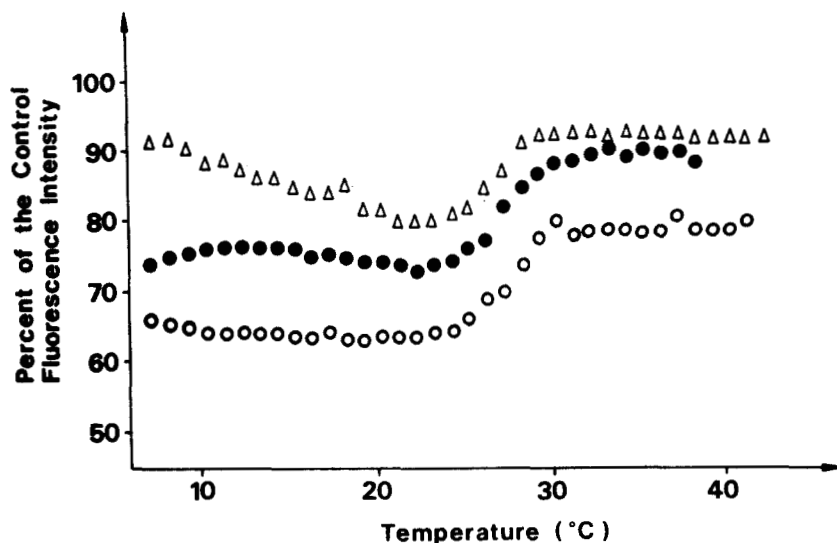

Fig. 7. Temperature-dependence on the tryptophan fluorescence quenching by *cis*-PnA (△), *trans*-PnA (●), and DPH (O). Lipophorin concentration was 300 μg of protein/ml and mole ratios of probe:lipophorin were 3:1 for all the probes. Excitation wavelengths were 280 nm and emission wavelengths were 340 nm. Temperature was increased at the rate of approximately 1°C/min, using a circulating water bath.
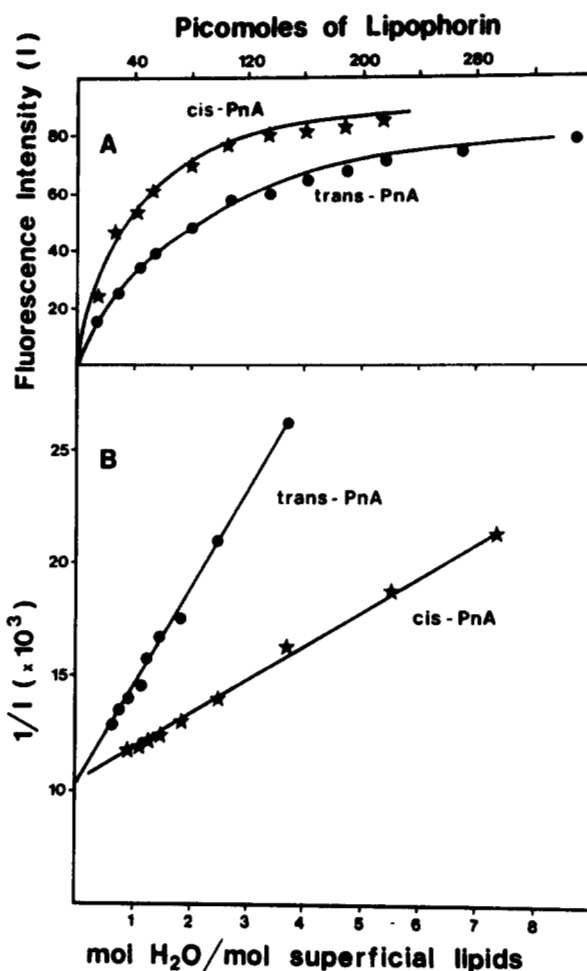
Fig. 8. The binding of *cis*-PnA and *trans*-PnA to lipophorin as determined by fluorescence enhancement at 6°C. A) Fluorescence intensity of parinaric acids. The concentration for *cis*-PnA was 1.5 µM and for *trans*-PnA 0.8 µM. Lipophorin was added as microliter aliquots from a stock solution of 5 mg of protein/ml. Fluorescence excitation was at 320 nm and emission was determined at 420 nm. B) Double reciprocal plot of I^{-1} mol of H_2O/mol of superficial lipids. It was assumed that the maximal fluorescence achieved when a large excess of lipophorin was added represented 100% binding, and that the amounts of PnA bound are proportional to their relative fluorescence intensities.

persion by fluorescence polarization of DPH. In addition, a gel phase formation at temperatures below 30°C has been recognized by fluorescence polarization of *trans*-PnA. Similarly, diacylglycerols showed a small gel phase formation below 30°C when the lipids were labeled with *trans*-PnA. Diphenylhexatriene failed to detect lipid transitions in phospholipid dispersions. However, *trans*-PnA allowed us to detect a small fraction of the phospholipids that formed a gel phase below 24°C. Differences found in *trans*-PnA and DPH breakpoints must be attributed to the properties of the probes. DPH has been shown to report the lowest critical temperature of the lipid transition (42, 43) while *trans*-PnA by its high solubility and quantum yield in gel phases (16, 44) allows detection of as little as 5% of the gel phase in the lipids. These properties of the

trans-PnA make it sensitive to the upper critical temperature of the transition.

Recently, Katagiri, Kimura, and Murase (9), employing differential scanning calorimetry to study the locust lipophorin, have reported two endothermic peaks centered at 11° and 36°C in both intact lipophorin and lipophorin hydrocarbon fractions. We have not extensively studied the hydrocarbon fraction of the *T. infestans* lipophorin, but it is probably involved in the lipid thermotropic transition of native lipophorin. However, the results obtained in the study of the diacylglycerol and in the phospholipid fractions allow us to suggest that a small fraction of these lipid classes can be involved in the lipid transition found at about 20°C.

Katagiri (8) has shown the superficial localization of the phospholipids in the lipoprotein particle of *Locusta* and although the distribution of the diacylglycerols in the lipophorin particle remains unknown, by their polar nature, it is valid to suppose that at least part of these lipids will be located superficially in the lipophorin. Moreover, taking into account the polar nature of the *trans*-PnA and *cis*-PnA and their superficial location in human lipoproteins (14, 15), we can propose that superficial lipophorin lipids are involved in the lipid transition found at about 20°C.

Because of their free hydroxyl group diacylglycerols have a larger polarity than triacylglycerols. Consequently, insect fatty acid transport can be carried out by superficial lipids of the lipoprotein while mammalian fatty acids
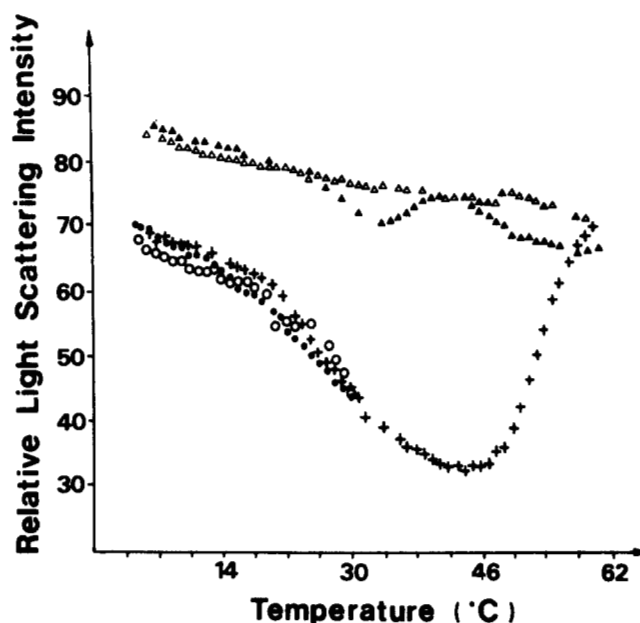

Fig. 9. Temperature-dependence on the 90° light scattering intensity. Lipophorin concentration was 300 µg of protein/ml in 50 mM potassium phosphate buffer, pH 7.2. Sample temperature was varied at approximately 1°C/min. The sequence in the experiment was: heating 5°→32°C (○); cooling 32°→5°C (●); reheating 5°→62°C (+); recooling 62°→6°C (△) and reheating 6°→32°C (▲).

must be transported in the core of the particle by the triacylglycerols. In addition, the superficial location of diacylglycerols would favor their exchange in the tissues, allowing the recycling of the lipophorin. Moreover, the solubility of diacylglycerols in lipid membranes would make unnecessary their breakdown to fatty acids and glycerol in order to be incorporated into the cell.

The large difference between the fluorescence polarization values of DPH or *trans*-PnA in the lipid dispersion and in the native lipophorin (Table 1) reflects the high lipid mobility restriction imposed by the apoproteins. These differences are larger than those found in human HDL (10) and could indicate the influence of the apoproteins in determining lipoprotein structure. This hypothesis is supported by the fact that diacylglycerols and phosphatidylethanolamine are the principal lipid components of the *T. infestans* lipophorin (1, 41), and moreover, that diacylglycerols represent an important fraction of the lipids in all the insect lipophorin studied (29). Phosphatidylethanolamine and diacylglycerols, due to the small size of their polar head groups, adopt hexagonal structures (H_{II}) (45–47). In addition, low concentrations of diacylglycerol induce lamellar-to-hexagonal structural transitions in phosphatidylethanolamine or phosphatidylcholine systems. Therefore, a globular lipophorin having a micellar structure with the head groups of the polar lipids in the surface of the spherical particle, can only be envisaged if the protein components exert a high interaction with DG and PE lipids, filling the free spaces left among the polar head groups by these molecules.

The location of the apoproteins on the surface of the particles, according to the preceding comments, is also supported by a previous report of Shapiro, Keim, and Law (48). These authors, employing double immunodiffusion tests, have found that in *Manduca sexta* both apoproteins of lipophorin, apoLp-I and apoLp-II are exposed to the aqueous environment. In addition, our results of the energy transfer experiments, which have shown a lesser tryptophan-probe average distance for the parinaric acid probes than for the DPH probe, are in agreement with a superficial location of the apoproteins (49).

Quenching curves of Fig. 7 show that at 24°C there is a decrease in the quenching efficiency for all the probes used. These results could be due, in part, to the expansion of the lipoprotein by a lipid transition and a corresponding decrease in the acceptor chromophore density. But it is also possible that a conformational change of the apoproteins could take place at this temperature. However, since thermotropic transitions of the lipoprotein lipids have been shown around this temperature, it is more probable that the change shown is due to a change in the order of the lipophorin lipids.

Light-scattering curves together with the gel filtration experiments showed that under our working conditions

lipophorin aggregation can be induced by effect of temperature. In a recent report, Chino et al. (28) presented some evidence supporting the proposal, previously mentioned by Wheeler and Goldsworthy (6) and Mwangi and Goldsworthy (50), that in the locust lipophorin O is not a naturally occurring component of the hemolymph. Thus, our results support the proposal that O lipophorin is produced by technical manipulation of the hemolymph. Moreover, the continuity found in the heating curves of the anisotropy of fluorescence of DPH (Fig. 2) above 44°C and the reversibility observed in the cooling curves suggest that no denaturation, or at least no change in the lipid–protein interactions, is produced by the particle aggregation. These results are in agreement with the results found with electronic microscopy by several authors (6, 28, 29, 51), where the called "O" lipophorin appears as a single aggregate of the lipophorin without extensive loss of the particle identity.

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