Purification and properties of the very high density lipoprotein from the hemolymph of adult *Triatoma infestans*

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Abstract The very high density lipoprotein (VHDL) of *Triatoma infestans* hemolymph from adult males has been isolated and purified by two-step density gradient ultracentrifugation. It appears to be homogeneous as judged by native polyacrylamide gel electrophoresis. The content of VHDL in hemolymph was estimated to be 8 mg protein/ml. The purified protein has a molecular weight (M_r) of 450,000, is composed of six subunits of $M_r \cong 77{,}000$, and possesses a high content of aromatic amino acids. This protein is glycosylated and contains 3% of lipids by weight with a remarkable amount of free fatty acids (25% of total lipids). The *T. infestans* VHDL has a different lipid and amino acid composition from lipophorin. The lipid composition and the spectroscopic studies using *cis*-parinaric acid indicated a high fatty acid binding affinity. It has nine binding sites per mol of VHDL. Competence studies revealed that VHDL has its highest affinity for the binding of palmitic acid followed by stearic and arachidonic acids.—**Rimoldi, O. J., J. L. Soulages, S. M. González, R. O. Peluffo, and R. R. Brenner.** Purification and properties of the very high density lipoprotein from the hemolymph of adult *Triatoma infestans*. *J. Lipid Res.* 1989. **30:** 857–864.

Supplementary key words insect lipoproteins • free fatty acid transport • insect VHDL • lipid-protein interactions

Different classes of lipoproteins have been characterized as constituents of the insect hemolymph. A specific and major class of lipoprotein referred to as lipophorin (1) is present in all life stages and in both sexes. This lipoprotein seems to be a multifunctional lipid carrier containing principally diacylglycerols, phospholipids, and other hydrophobic materials. Lipophorins exist as dynamic molecules and the changes in particle size and density may be correlated to the physiological or developmental state of the insect. Research on lipophorin has been documented in recent reviews (2, 3).

A second class of hemolymph lipoprotein, known as vitellogenin, is the predominant yolk protein precursor, produced in the fat body, transported in the hemolymph, and taken up by the growing oocytes (4). Vitellogenins are generally restricted to reproductive female insects, although very low concentrations are found in the hemolymph of some male insects (2).

In recent studies, very high density lipoproteins or VHDL have been reported in *Heliotis zea* (5) and *Apis mellifera* (6). They are absent during all early larval stages in pupae and in adult insects, but are the major hemolymph proteins in 5th instar larvae. These lipoproteins possess a developmental pattern similar to that of storage proteins. Although their physiological function remains unclear at the present time, Shipman et al. (6) and Haunerland and Bowers (5) suggested a potential role in lipid transport and/or storage of proteins during the metamorphosis.

In a previous study (7) of the hemolymph lipoproteins of the adult male *Triatoma infestans,* a hemimetabolous insect, we reported the existence of three classes of lipoproteins: an HDL (also called lipophorin) and two VHDLs with densities 1.19–1.23 and 1.25–1.26 g/ml, respectively. In addition, a fourth lipoprotein was found in female hemolymph that can be identified as a vitellogenin (8). Subsequent investigations have fully explored the composition and the physicochemical properties of lipophorin in this insect (9).

The purpose of the present work was to purify the VHDL fraction of d 1.25–1.26 g/ml and to examine its composition and physical and chemical characteristics. It is suggested that it has a possible functional role in the lipid transport.

MATERIALS AND METHODS

Insects

Adult male *T. infestans,* reared on hen blood twice a week and maintained at 28°C and 60–70% humidity in our laboratory, were used for the experiments. Seven or

Abbreviations: VHDL, very high density lipoprotein; TLC, thin-layer chromatography; FID, flame ionization detection; GLC, gas–liquid chromatography; PAGE, polyacrylamide gel electrophoresis; *cis*-PnA, *cis*-parinaric acid.

8 days after the last moulting, the insects were fed once and then fasted for 5 days prior to hemolymph collection.

Hemolymph collection

To collect hemolymph the insects were anesthetized by cooling on ice. The legs were cut off and the insects were placed in a precooled centrifuge tube with the head toward the pointed end and centrifuged at 120 *g* at 4°C for 1 min. The hemolymph was pooled into a precooled tube and centrifuged at 10,000 *g* at 4°C for 10 min to remove hemocytes. Darkening of hemolymph by air exposure was prevented by addition of 5 mM N-acetyl-L-cysteine.

Purification of VHDL

Hemolymph was ultracentrifuged to fractionate the lipoproteins using a procedure similar to that described by Fichera and Brenner (7). Three ml of NaBr solution (density 1.28 g/ml) was placed in a centrifuge tube and 1 ml of the hemocyte-free hemolymph was carefully layered over the solution and centrifuged at 178,000 *g* at 10°C for 20 hr in a Beckman L8 70M ultracentrifuge with an SW 60 Ti rotor. The tube contents were then fractionated from the top into 0.2-ml fractions. After dialysis to remove NaBr the fractions were analyzed by native polyacrylamide gel electrophoresis (native-PAGE) to determine the fractions containing VHDL. The peak fractions were pooled, and NaCl solution (d 1.006 g/ml) was added up to 1 ml. This mixture was layered over 3 ml NaBr solution (d 1.31 g/ml), and recentrifuged and fractionated as described above. A blank tube without VHDL (1 ml NaCl solution, d 1.006 g/ml) was centrifuged with sample tubes and the density of successive 0.2-ml fractions collected from the top was determined by refractometry.

The VHDL-containing fractions were pooled and extensively dialyzed against 0.02 M sodium phosphate buffer (pH 7.2), 130 mM NaCl, 1 mM EDTA, and 0.02% NaN_3 at 4°C.

Gel permeation chromatography

The purified VHDL was applied to a 80 × 1.6 cm column packed with Sephacryl-S 300 (Pharmacia, Uppsala, Sweden) at room temperature and eluted with the same solution used in the dialysis. The flow rate was 20 ml/hr and 2-ml fractions were collected. The elution pattern of the proteins was determined by monitoring the eluent at 280 nm. Blue dextran (Pharmacia) served as marker of void volume (Vo). The column was calibrated for molecular weight using thyroglobulin, ferritin, catalase, and aldolase as standards (Pharmacia). A least-square regression of log molecular weight versus (Ve – Vo)/(Vt – Vo) (in which Ve is the elution volume of the protein, Vo and Vt are void and total bed volumes, respectively) was used to calculate the molecular weight of the VHDL fraction.

Polyacrylamide gel electrophoresis

Native gel electrophoresis (native-PAGE) was performed on 4–20% acrylamide gradient slab gels. The resolving gel (in 0.375 M Tris-HCl, pH 8.8) was overlayered with a 3.75% stacking gel containing 0.125 M Tris-HCl, pH 6.8; the electrode buffer contained Tris–glycine, pH 8.3 (0.025 M Tris, 0.192 M glycine).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (10) using a 4–20% acrylamide gradient gel.

Proteins were visualized by staining with Coomassie brilliant blue, followed by destaining of the gel background.

The native and subunit molecular weights were determined by comparison with concurrently electrophoresed standard proteins of known molecular weight (Pharmacia, High and Low Molecular Weight Kits). The regression line of log molecular weight as a function of distance travelled by the standards was calculated by the method of least squares.

Amino acid analysis

Amino acid composition of VHDL was determined on duplicate samples hydrolyzed in 6 M HCl in vacuo at 110°C for 24 h. Cysteine and cystine residues were oxidized to cysteic acid by the method of Hirs (11). Analyses were performed on a Beckman 119 CL amino acid analyzer.

Protein and carbohydrate determination

Protein concentration in the VHDL fraction obtained after two-step density gradient ultracentrifugation was quantified by the method of Lowry et al. (12) using bovine serum albumin as standard.

The presence of covalently bound carbohydrate in VHDL apoprotein was detected by periodate-Schiff staining on an SDS-PAGE slab gel following the technique of Zacharius et al. (13).

Lipid analysis

A known amount of lipoprotein was extracted with chloroform–methanol 2:1 according to Folch, Lees, and Sloane Stanley (14). The solvent was removed from the extract under a stream of N_2 and the lipid content was determined gravimetrically. Phospholipids were measured by the method of Chen, Toribara, and Huber (15). Quantitation of individual neutral lipids was achieved by thin-layer chromatography–flame ionization detection (TLC–FID) system. A comprehensive description of this technique is given by Ackman (16). FID scans were performed on the Iatroscan TH-10 analyzer (Iatron Laboratories, Tokyo, Japan) with a LKB 2210 dual pen strip chart recorder (LKB Produkter AB, Bromma, Sweden). The FID was operated with hydrogen pressure of 0.50 kg/cm^2

and air flow rate of 2 l/min. Chromarods (type S) were spotted with 20 μg of lipid in chloroform solution and were developed first in hexane–diethyl ether–formic acid 98:2:0.2. Following development, the rods were dried at room temperature for 15 min and scanned till the lowest point on the tail of the free fatty acid peak. The remaining unpyrolyzed lipid classes were developed with hexane–diethyl ether–formic acid 80:20:0.2. Then, the rods were dried at 120°C for 4 min and fully scanned. Peak areas were measured with a stepping pen integrator and converted to weight percent lipid class by the use of correction factors derived from internal standard (methyl docosanoate) calibration curves performed for each individual lipid.

Fatty acid composition

Lipid classes present in the VHDL were separated by thin-layer chromatography (TLC) on silica gel G plates developed in hexane–diethyl ether–acetic acid 80:20:2. Appropriate standards were visualized by exposure to iodine vapor. The unexposed sample bands corresponding to free fatty acids and diacylglycerols were scraped from the plate, extracted with methanol–chloroform 2:1 and esterified with 3 M HCl in methanol at 64°C for 3 h. The fatty acid methyl esters were analyzed by gas–liquid chromatography (GLC) in a Hewlett-Packard 5840 A apparatus with a 6-ft column packed with GP 10% Sp 2330 on 100–120 Chromosorb WAW (Supelco Inc., Bellefonte, PA). The temperature program was 140–220°C at 3°C/min. Individual methyl ester peaks were identified using pure standards (Nu-Chek Prep, Elysian, MN). Relative composition was obtained by area normalization directly from the Hewlett-Packard 5840 A terminal.

Binding studies

Cis-parinaric acid (*cis*-PnA) was obtained from Pharmacia (PL-Biochemicals). It was stored as an ethanolic solution containing 1% of BHT at −20°C. The binding of *cis*- PnA to VHDL was determined by fluorescence enhancement according with Sklar et al. (17, 18). Two ml of sodium phosphate buffer (50 mM), EDTA (5 mM), pH 7.4, containing *cis*-PnA (2×10^{-6} M) was transferred to the cuvette. Purified VHDL was added in microliter aliquots from a stock solution of 16 μg protein/μl. Fluorescence intensity (I) was measured with an Aminco Bowman spectrofluorometer. Excitation of *cis*-PnA was performed at 320 nm and emission was measured at 420 nm. In order to avoid photochemical damage of *cis*-PnA, the probe was always added after buffer deoxygenation by vigorous stirring under N_2, and the fluorescence excitation was performed for a few seconds using a cell slit of 0.1 mm. Furthermore, no significant differences were found between the final intensity recorded after the addition of successive aliquots of VHDL and the intensity obtained after a sole addition of an equivalent amount of VHDL. On this basis, it is concluded that no damage of *cis*-PnA resulted from the experiment. The temperature of the assay system was controlled by a circulating water bath and measured with a thermocouple.

The partition constant K_p defined as:

$$K_p = \frac{\text{moles of bound PnA/mole of VHDL}}{\text{moles of free PnA/mole of } H_2O}$$

was obtained from the slope of the double reciprocal plot of I^{-1} against mol H_2O/mol of VHDL. It is based on the equation:

$$\frac{1}{I} = \frac{1}{I_{max}} + \frac{1}{I_{max}\,K_p} \cdot \left(\frac{\text{moles } H_2O}{\text{mole VHDL}}\right)$$

where I_{max} is the maximal fluorescence intensity of *cis*-PnA calculated from the y intercept.

RESULTS AND DISCUSSION

VHDL purification

The VHDL fraction was isolated and purified from hemolymph of adult male *T. infestans* by two successive ultracentrifugations in different density gradients. Nineteen fractions separated on the basis of density were collected after the preliminary ultracentrifugation and were analyzed by native-PAGE. As shown in **Fig. 1,** VHDL (lanes 14–19) was separated from the floating lipophorin (lanes 1–6) and from the heterogeneous protein fraction (lanes 7–13). However, some contaminants remained in the VHDL fractions composed of lower molecular weight proteins. For this reason, the fractions enriched in VHDL were pooled, the density was adjusted by dialysis and subjected to a second ultracentrifugation. After this second step, the analysis of the major protein peak by native-PAGE demonstrated that VHDL was electrophoretically pure (**Fig. 2**).

The equilibrium density of the VHDL particle was determined to be 1.27 g/ml from comparison with the refractive index of reference blank tubes.

Molecular weight and protein composition

This lipoprotein has a native molecular weight of about 450,000 as determined by gradient gel electrophoresis (native-PAGE). It was estimated by plotting the log molecular weight of a set of marker proteins versus their relative migration on the gel. The marker proteins used were tyroglobulin, ferritin, catalase, lactate dehydrogenase, and albumin. On the other hand, the molecular weight determined by gel permeation chromatography on Sephacryl-S300, as described in Materials and Methods, was approximately 390,000. The slightly lower molecular weight estimated by gel permeation chromatography may be explained by interactions between glycoproteins and

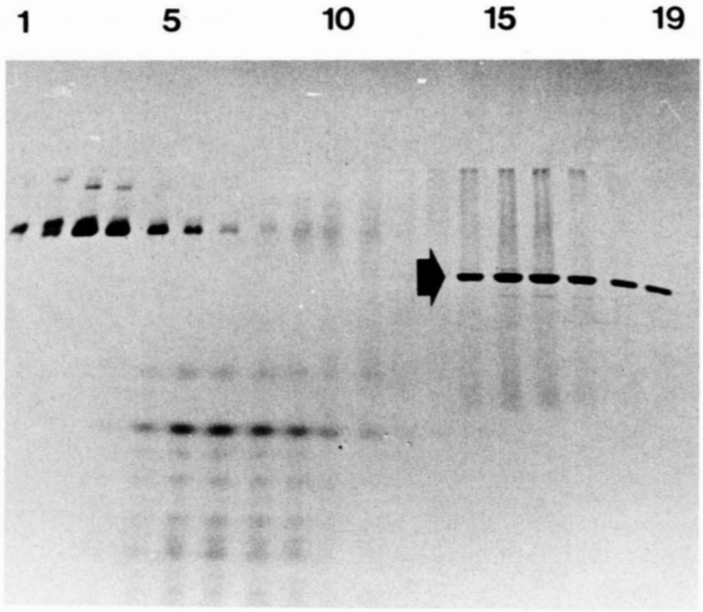

Fig. 1. Native polyacrylamide gel electrophoresis of the preliminary density gradient ultracentrifugation fractions of *T. infestans* hemolymph. Fractions obtained after centrifugation (0.2 ml) were dialyzed and loaded onto a 4–20% polyacrylamide gradient slab (lanes 1–19). Proteins were stained with Coomassie blue. Arrow indicates position of VHDL.

the dextran matrix (19). Electrophoresis of the VHDL fraction under denaturing conditions revealed that it comprises only one protein component with an apparent molecular weight of ≅77,000 (**Fig. 3**). These data taken together seem to indicate a hexameric subunit structure for native VHDL. A similar VHDL-apoprotein was detected in preliminary work (20).

The protein is also glycosylated as visualized by periodate-Schiff staining on SDS-PAGE.

The amino acid composition of the VHDL apoprotein is summarized in **Table 1.** It shows about 40% nonpolar amino acids. The noteworthy features of the amino acid composition are the high content of aspartate, glutamate, and aromatic amino acids (tyrosine and phenylalanine) and the relative paucity of methionine and cysteine residues. It is important to note that VHDL apoprotein composition differs from that of *T. infestans* lipophorin apoproteins. Lipophorin is comprised of two apoproteins ApoLp-I (mol wt 255,000) and ApoLp-II (mol wt 78,000) (Rimoldi, O. J., J. L. Soulages, M. S. González, R. O. Peluffo, and R. R. Brenner, unpublished observations) whereas the only monomeric apoprotein of VHDL has an apparent molecular weight of 77,000. In spite of the molecular weight similarity between ApoLp-II and VHDL apoprotein, the amino acid composition differs in several amino acid proportions, principally alanine, tyrosine, and phenylalanine.

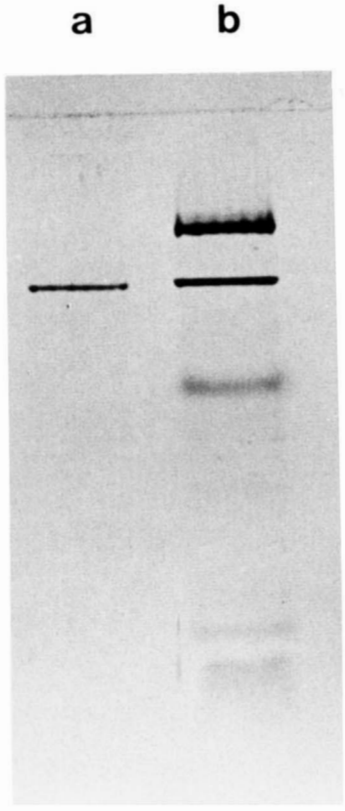

Fig. 2. Native polyacrylamide gel electrophoresis of *T. infestans* VHDL isolated and purified after two-step density gradient ultracentrifugation (lane a) and whole hemolymph (lane b). The samples were applied to a 4–20% polyacrylamide gradient slab.

Lipid composition and possible function of VHDL

Lipids comprise about 3% of lipoprotein mass. Further characterization and quantitative analysis of the lipid

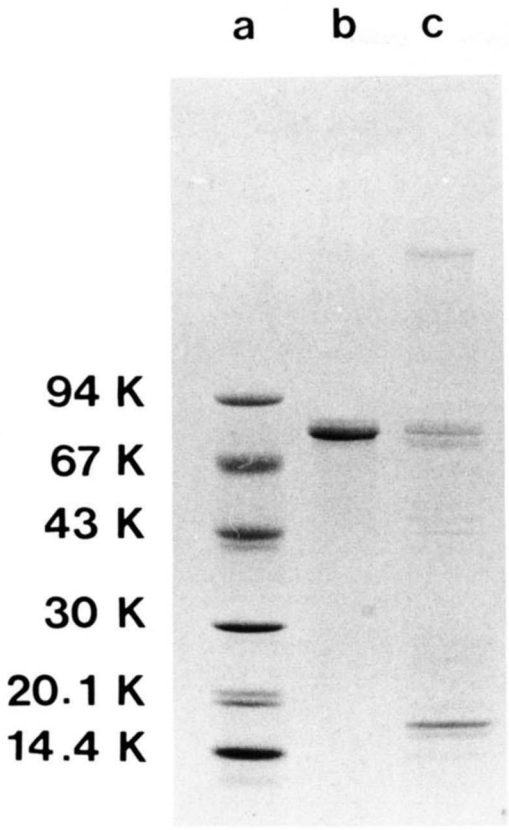

Fig. 3. SDS-polyacrylamide gel electrophoresis of purified *T. infestans* VHDL. A 4–10% polyacrylamide gradient slab was used. Lane a, molecular weight standards: phosphorylase b (M_r = 94,000), bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 43,000), carbonic anhydrase (M_r = 30,000), trypsin inhibitor (M_r = 20,100), and α-lactalbumin (M_r = 14,400); lane b, purified VHDL and lane c, whole hemolymph.

components (**Table 2**) showed a large percentage of polar lipids (59% of the total lipids) and a strikingly high content of free fatty acids (25%). Diacylglycerol, triacylglycerol, hydrocarbon, and cholesteryl ester were low. In a previous study, Fichera and Brenner (7) found a lower content of free fatty acids and a higher content of hydrocarbons in VHDL. However, these differences must be attributed to the partially purified VHDL used in that preliminary study. The fatty acid composition of the free fatty acid and diacylglycerols was determined, and the results are shown in **Table 3.** This analysis revealed that about 65% of the total free fatty acids are saturated, with palmitic and stearic acids as the main components; linoleic, oleic, and arachidonic acids are the principal unsaturated fatty acids. The high content of palmitic acid (39.0%) is remarkable.

The VHDL diacylglycerols represent a small percentage of the total hemolymph diacylglycerols and their fatty acid composition shows some differences with respect to the composition of diacylglycerols carried by lipophorin (Table 3). Lipophorin diacylglycerols have a high content

TABLE 1. Amino acid composition of *T. infestans* VHDL apoprotein

Amino Acid	Mol%	Residues/Mol
Aspartate[a]	14.2	89
Threonine	4.3	27
Serine	4.2	26
Glutamate[a]	9.9	62
Proline	4.9	31
Glicine	6.2	39
Alanine	5.6	35
Valine	7.7	48
Methionine	0.2	1
Isoleucine	3.3	21
Leucine	9.5	59
Tyrosine	7.1	44
Phenylalanine	7.0	44
Histidine	2.4	15
Lysine	7.0	44
Arginine	5.4	34
Cysteine[b]	1.0	6
Tryptophan	n.d.	n.d.
Total	99.9	625

[a]Includes acids + amide.
[b]Determined as cysteic acid after performic acid oxidation.
n.d., Not determined.

of oleic acid (52.8%) and about 38% of saturated fatty acids. On the other hand, VHDL diacylglycerols have a low content of oleic acid (14.2%) and about 55% of saturated fatty acids.

It should be noted that the VHDL lipid fractions had a significant content (~7%) of an acid with the same retention time as that of arachidonic acid. Its proportion was even higher than that of linoleic acid.

From the molecular weight, the hexameric structure, and the amino acid composition, *T. infestans* VHDL could be envisaged as an arylphorin (19, 21, 22). However, the arylphorins have been found in the hemolymph of larvae and pupae, but are absent in the adult stage. The fact that VHDL is present in the adult stage apparently discards the possibility that it can serve as a storage form for amino acids, which is the principal role attributed to arylphorins (23, 24). On the other hand, while lipid analysis of *T. infestans* VHDL indicated that free fatty acids are the major

TABLE 2. Relative percentage composition of lipid fractions in *T. infestans* VHDL

Lipid Fractions	%
Polar lipids	59.0 ± 3.5
Free fatty acids	24.9 ± 1.5
Diacylglycerols	5.5 ± 0.9
Triacylglycerols	3.7 ± 0.6
Cholesterol	trace
Cholesteryl esters	2.3 ± 0.2
Hydrocarbons	4.6 ± 0.8

Values are means ± SD (n = 3).

TABLE 3. Fatty acid composition of the major lipid classes of *T. infestans* VHDL and lipophorin

Fatty Acid	VHDL		Lipophorin
	Free Fatty Acids	Diacylglycerols	Diacylglycerols
14:0	6.7 ± 0.5	7.3 ± 0.6	
16:0	39.0 ± 1.7	34.4 ± 1.5	33.1 ± 1.3
16:1	6.5 ± 0.5	11.6 ± 0.8	2.7 ± 0.3
18:0	19.1 ± 0.9	13.8 ± 0.8	5.3 ± 0.3
18:1	10.0 ± 0.7	14.2 ± 0.6	52.8 ± 1.1
18:2	6.6 ± 0.7	6.0 ± 0.5	6.1 ± 0.5
20:3	4.3 ± 0.4	5.6 ± 0.4	
20:4 (n-6)	7.8 ± 0.8	7.1 ± 0.5	
Total saturated	64.8	55.5	38.4
Total unsaturated	16.5	25.8	55.5
Total polyunsaturated	18.7	18.7	6.1

The results are the mean of four samples ± SD and are expressed as percentages of the total fatty acids.

neutral lipid, diacylglycerols and monoacylglycerols are the principal components of arylphorin neutral lipids (25).

The lipid classes and their relative amounts found in *T. infestans* VHDL were similar to those of larval-specific VHDL recently isolated and characterized from the hemolymph of the honeybee *Apis mellifera* (6). However, there are large differences between these two VHDLs with respect to their molecular weights and oligomeric structures.

From the *T. infestans* VHDL composition, we think that one of the most important roles of this fraction may be the transport of free fatty acids. To obtain further support for this hypothesis, we measured the level of free fatty acid in hemolymph and the concentration of VHDL. The free fatty acid level was 0.31 mg/ml hemolymph. Similar values have been reported in other resting insects (2). The VHDL concentration was ca. 8 mg/ml hemolymph. Thus, the content of VHDL free fatty acid suggested that VHDL may participate in the physiological transport of this lipid.

Fatty acid binding properties of VHDL

In order to further specify the ligand binding properties of VHDL, we have used fluorescence enhancement methods to measure binding of fatty acids to VHDL. Several spectroscopic methods have been employed to determine binding of fatty acids to proteins (17, 18, 26). For these studies, the properties of the natural polyene fatty acid, *cis*-PnA, as spectroscopic probe of lipid–protein interactions, were utilized.

The enhancement of the fluorescence of a solution of *cis*-PnA to which VHDL was added is shown in **Fig. 4a.** The initial linear increase in fluorescence intensity intercepted the maximal fluorescence intensity (obtained from the y intercept of the double reciprocal plot of Fig. 4b) at a value close to 0.11 mol of VHDL per mol of *cis*-PnA, which indicated that about nine fatty acid binding sites can be occupied under these conditions.

A double reciprocal plot of I^{-1} versus mol H_2O/mol VHDL may be derived from the titration curve of Fig. 4a. The partition constant of *cis*-PnA between VHDL and water was determined from the slope of the plot (Fig. 4b) (17). As determined by regression analysis, the data best fit a two-slope plot. Two K_p values were obtained in this

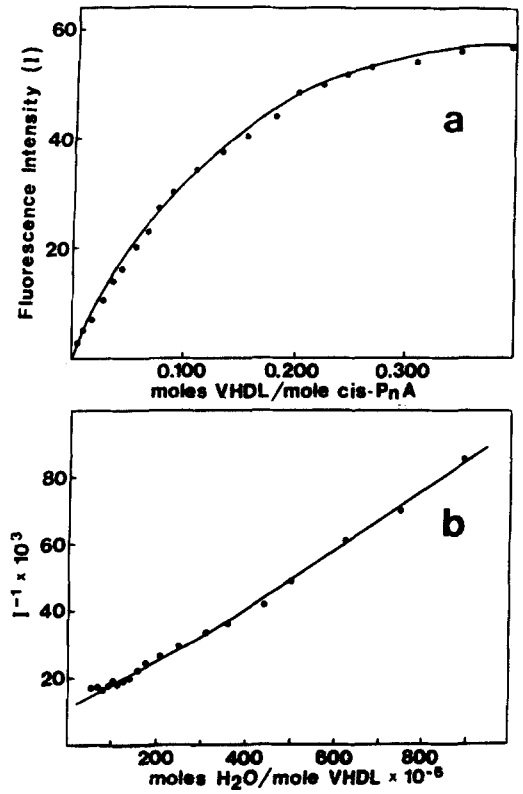

Fig. 4. Binding of *cis*-PnA to VHDL. a) Fluorescence intensity of a solution of 2 μM *cis*-PnA. VHDL was added as microliter aliquots from a stock solution. b) A double reciprocal plot of I^{-1} versus mol H_2O/mol of VHDL. It was assumed that the maximal fluorescence intensity (y intercept) achieved when a large excess of VHDL was added represents 100% binding.

way, K_{p_1} of about 290 × 10^6 and K_{p_2} 180 × 10^6, which indicated a high average fatty acid binding affinity of VHDL.

Fatty acid binding competition for VHDL

In order to compare the free fatty acid composition of VHDL with its binding properties, we studied the competition between *cis*-PnA and several other fatty acids, as shown in **Fig. 5.** The fatty acid under study was added to a 0.1 μM solution of VHDL in a 25 molar ratio of free fatty acid to VHDL. The displacement of fatty acids by *cis*-PnA was recorded by enhancement of fluorescence intensity. The data shown in Fig. 5 indicated that arachidonic acid was more easily displaced by PnA than stearic and palmitic acids. The relative affinity of VHDL for these free fatty acids is 16:0 > 18:0 > 20:4. To quantify the affinity constant differences found among these fatty acids, we developed a simple approximation (see Appendix) by which the partition constant ratio between two fatty acids may be obtained. In this way the following values were found:

$$K_{16:0}/K_{20:4} = 2.1 \text{ and } K_{16:0}/K_{18:0} = 1.5$$

It is interesting to note that the fatty acid binding affinity of VHDL is consistent with the VHDL free fatty acid composition.

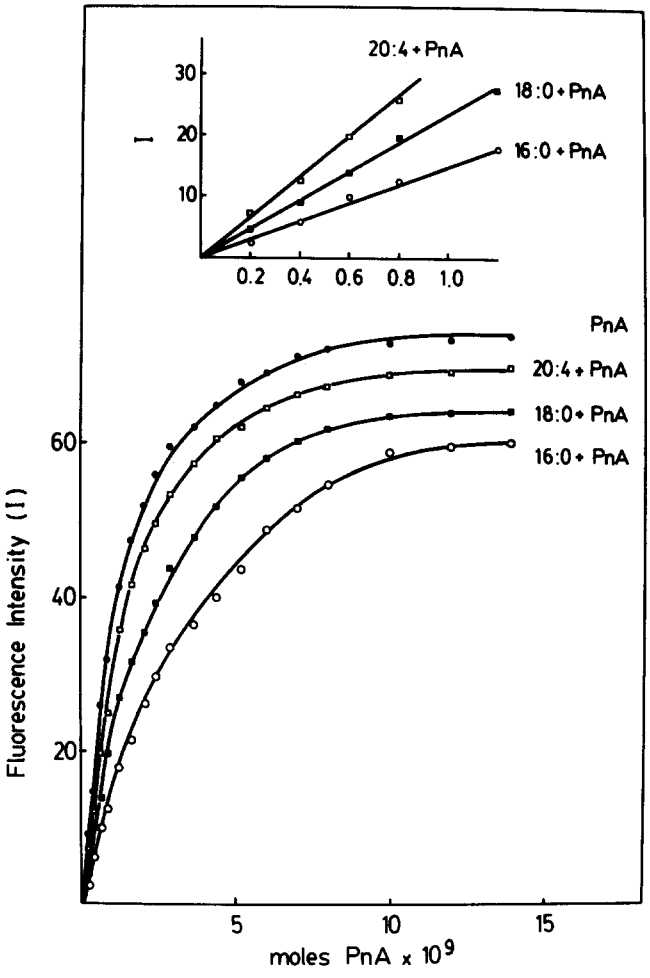

Fig. 5. Binding competition between *cis*-PnA and several fatty acids for VHDL. VHDL (0.2 nmol) was incubated with 5 nmol of the tested fatty acid. Aliquots of an ethanolic solution of *cis*-PnA were added, and the displacement of fatty acids by *cis*-PnA was recorded by the enhancement of the fluorescence. The inset shows the first part of the curve in an amplified form. This plot was used to determine the relative affinity constants among the fatty acids tested.

Metabolic importance of free fatty acids

Since hemolymph free fatty acids are present in relatively small amounts, their metabolic significance has remained unrecognized up to now. Recently, we estimated that the half-life of the *T. infestans* hemolymph free fatty acid was around 4 min (27), a value similar to that found for mammals (28). No additional information on free fatty acid half-life could be found in the available literature for other insects. Nevertheless, published results (29) on the half-life of the hemolymph diacylglycerols from locust under conditions of rest indicate a value of about 2.5 h. Then, although the hemolymph diacylglycerol level exceeds that of free fatty acids by a factor of 10, the contribution of fatty acids to the tissues due to the hemolymph free fatty acids may be higher than that of diacylglycerols. We have shown that approximately 65% of the VHDL free fatty acids are saturated. Thus, it may be valid to suppose that, to a great extent, VHDL free fatty acids can be used as an energy source. However, VHDL has a relatively high content of arachidonic acid which probably renders it able to transport this prostaglandin precursor (30, 31).

In brief, the major findings and suggestions of the present study are: *a*) VHDL is a glycoprotein found in high levels in adult *T. infestans* hemolymph; *b*) it contains different lipid classes, but an unusually high content of free fatty acids is a hallmark of this lipoprotein; and *c*) this fraction may play an important physiological role as a hemolymph free fatty acid carrier.

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APPENDIX

The equilibrium constant ratio between PnA and fatty acid A is:

$$\frac{K^{PnA}}{K^{A}} = K_r^{A} = \frac{A_f \times PnA_b}{A_b \times PnA_f} \qquad Eq.\ 1$$

where the subscripts b and f represent the bound and free forms, respectively. In equations 1-4, superscripts A and B refer to the respective fatty acid in the system. If the total concentration of the fatty acid, A, is several-fold larger than the protein (P), the

protein will be saturated and $A_b = N[P]$ and $A_f = A_t - A_b = A_t - N[P]$, where N is the total number of binding sites. If $PnA_t = A_t$, the concentration of free PnA may be considered equal to the total PnA concentration ($PnA_f = PnA_t$) and equation 1 may be expressed:

$$K_r^A = \frac{(A_t - N[P]) \times PnA_b^A}{N[P] \times PnA_t^A}. \qquad Eq.\ 2$$

In a similar way for a fatty acid B, we have

$$K_r^B = \frac{(B_t - N[P]) \times PnA_b^B}{N[P] \times PnA_t^B}. \qquad Eq.\ 3$$

Dividing equation 3 by equation 2 in conditions where $PnA_b^A = PnA_b^B$, that is at the same fluorescence intensity, we obtain:

$$K^{A/B} = \frac{PnA_t^A}{PnA_t^B} \qquad Eq.\ 4$$

where the total concentrations of A and B as well as protein were the same as those used in our experiments. Therefore, the equation indicates that the ratio of the total concentration of PnA needed to obtain the same fluorescence intensity, under these experimental conditions, is the relative partition constant between the A and B fatty acids.

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