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Characterization of lipophorin binding to the midgut of larval *Manduca sexta*

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Abstract

Lipophorin binding to the midgut of *Manduca sexta* larvae was characterized in a midgut membrane preparation, using iodinated larval high-density lipophorin (¹²⁵I-HDLp-L). The iodination procedure did not change the affinity of the preparation for lipophorin. In the presence of increasing concentrations of membrane protein, corresponding increases in lipophorin binding were observed. The time-course of lipophorin binding to the membranes was affected by the lipophorin concentration in the medium, and at a low lipoprotein concentration, a longer time was required for equilibrium to be reached. The specific binding of lipophorin to the midgut membrane was a saturable process with a $K_d=1.5\pm 0.2\times 10^{-7}$ M and a maximal binding capacity= 127 ± 17 ng lipophorin/ μ g of membrane protein. Binding did not depend on calcium, was maximal around pH 5.5, was strongly inhibited by an increase in the ionic strength, and abolished by suramin. However, suramin did not completely displace lipophorin that was previously bound to the membrane preparation. The lipid content of the lipophorin did not significantly affect the affinity of the membrane preparation for lipoprotein. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Lipophorin; *Manduca sexta*; Midgut; Lipoprotein

1. Introduction

Several classes of lipids—diacylglycerol, phospholipids, cholesterol, and hydrocarbons—are transported in insect hemolymph by lipophorin, the major hemolymph lipoprotein (Chino et al., 1981; Chino, 1985; Shapiro et al., 1988; Soulages and Wells, 1994; Blacklock and Ryan, 1994). All lipophorins contain two apolipoproteins, apolipophorin-I and -II (apoLp-I and apoLp-II), and the adults of some species contain a third apolipoprotein, apoLp-III (Kanost et al., 1990; Van der Horst, 1990; Soulages and Wells, 1994). Lipophorin particles can contain different amounts of lipids and are classified

according to their densities as HDLp (high density lipophorin), LDLp (low density lipophorin) or VHDLp (very high density lipophorin) (Beenackers et al., 1988). In some species, such as *Manduca sexta* and *Locusta migratoria*, the lipophorin density depends on the life stage, as well as, the physiological situation of the insect (Prasad et al., 1986; Surholt et al., 1992).

During lipid delivery to different tissues, lipophorin apolipoproteins are not accumulated or degraded in the organs, and can be reloaded with more lipids and reutilized, thus lipophorin acts as a reusable lipid shuttle (Downer and Chino, 1985; Van Heusden et al., 1987). It has been shown that lipophorin interaction with tissues is mediated by specific binding sites (Van Antwerpen et al., 1989; Atella et al. 1992, 1995), and in some experiments it seems that lipid transfer occurs at the surface of the cells, without the particle internalization (Van Antwerpen et al., 1988; Machado et al., 1996). These data have led to the suggestion that lipophorin binds to the cell surface via a docking receptor, but not much is known about these putative receptors. A lipophorin receptor from the fat body of *M. sexta* larvae was isolated and characterized (Tsuchida and Wells, 1990), and

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the fat body receptor from *L. migratoria* was also studied (Dantuma et al., 1996). On the other hand it has been shown that lipophorin can be internalized into midgut (Bauerfeind and Komnick, 1992) and fat body (Dantuma et al., 1997, 1999). Such observations might suggest the existence of a modified endocytotic cycle (Dantuma et al., 1998).

In feeding *M. sexta* larvae, lipophorin cycles between the digestive tract, where lipids are absorbed from the diet and transferred to the circulating lipoprotein, and the fat body, where the lipids are taken up and stored (Tsuchida and Wells, 1988). However, no lipophorin receptor from the digestive system has been studied. In this paper, the binding of lipophorin to the midgut of larval *M. sexta* was characterized in order to understand more completely the lipid transport system in this insect.

2. Materials and methods

2.1. Animals and hemolymph collection

M. sexta were raised as previously described (Prasad et al., 1986; Fernando-Warnakulasuriya et al., 1988). For one experiment, insects were raised from hatching on a high-fat diet (Fernando-Warnakulasuriya et al., 1988). Hemolymph was collected through an incision in the second pair of prolegs into PBS (phosphate-buffered saline), which is 12 mM potassium phosphate, pH 6.7, containing 2.5 mM EDTA, 5 mM glutathione, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM benzamidine. Hemolymph was collected from insects at the end of the fifth instar—for normal insects this is day 4 and for insects raised on a high fat diet this is day 6—and from first day normal wanderer larvae (prepupal stage). After centrifugation of the hemolymph for 20 min in a clinical centrifuge, the supernatant was collected for lipophorin purification.

2.2. Lipophorin purification

To purify lipophorin from normal feeding fifth instar larvae (HDLp-L), the collected hemolymph was adjusted to 44.5% KBr and 12 ml of this solution was overlaid with the same volume of 15% KBr in the same buffer and centrifuged in a Beckman 60 Ti rotor at 50,000 rpm for 16 h at 4°C. In this way at least two lipophorin populations could be separated. The one found in the 2.5 ml at the top of the gradient was discarded and the following 3.0 ml, containing the major lipophorin population (density 1.138 ± 0.003 g/ml), was collected and subjected to a second identical centrifugation. Sometimes, a third, denser population was also observed after the first gradient and it was also discarded. The existence of more than one lipophorin population in larval *M. sexta* hemolymph has been previously observed (Ziegler et al., 1995). For

the purification of lipophorin from first day wanderer larvae the same procedure was used, except that the hemolymph solution containing 44.5% KBr was overlaid with 22.5% KBr. This change in density of the overlaying KBr solution moved the lipophorin band to a higher position in the tube and gave a purer preparation, as determined by SDS-PAGE. At least two lipophorin populations were again observed (Ziegler et al., 1995), and the denser one (high-density lipophorin-wanderer 2, HDLp-W₂) was obtained from the 3.0 ml localized 2.5 ml from the top of the gradient. This fraction was then subjected to a second identical centrifugation. The same ultracentrifugation procedure was also used for the purification of lipophorin from the insects raised on the high-fat diet, except the 44.5% KBr solution was overlaid with 5% KBr. Again the density of the overlaying KBr solution was chosen to position the lipophorin band in the tube in such a way as to maximize purification. After centrifugation, one lipophorin population was observed, and it was subjected to a second identical centrifugation.

For the purification of lipophorin from oocytes (very high density lipophorin-egg, VHDLp-E), approximately 20 g of chorionated oocytes, dissected from adult females, were ruptured using a mortar and pestle, and homogenized with a Polytron homogenizer in 40 ml of PBS (100 mM sodium phosphate, 0.15 M NaCl, 5 mM EDTA, pH 7.0), containing 1 mM PMSF, 5 mM benzamidine, 100 μM leupeptin and 1 μM pepstatin A. The material was centrifuged for 1 h at 100,000 g at 4°C in a Beckman 60 Ti rotor. VHDLp-E was then purified from the supernatant, by means of two consecutive KBr ultracentrifugation gradients, as described by Kawooya et al. (1988).

The purified lipophorins were extensively dialyzed against 5 mM Tris, 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 0.15 M NaCl, pH 6.5, frozen in liquid nitrogen and stored at -80°C. The degree of purification was monitored by polyacrylamide gel electrophoresis in the presence of SDS (Laemmli, 1970).

2.3. Lipophorin iodination

Purified HDLp-L was iodinated with ¹²⁵I-sodium iodide (17.4 Ci/mg, DuPont-New England Nuclear, Wilmington, DE), in the proportion of 200 μCi/mg of protein, using Iodo-gen (Pierce, Rockford, IL) (100 μg/mg of protein), following the manufacturer's instructions. To remove the free iodide, the reaction mixture was passed through three pre-packed desalting PD-10 columns (Pharmacia Biotech, Uppsala, Sweden). For a control experiment, HDLp-L was also iodinated with non-radioactive NaI, following exactly the same procedure used for the radioactive labeling.

2.4. Midgut membrane preparation

Midguts were dissected from 20 fifth instar larvae (day 4), and the Malpighi tubules and peritrophic membranes were removed. After washing, the dissected organs were kept in cold insect saline (Jungreis et al., 1973), in the presence of 0.5 mM PMSF. The midguts were homogenized (20 times, Polytron homogenizer) in 20 ml of cold buffer (10 mM Tris, 5 mM MOPS, 0.15M NaCl, pH 6.5, 0.5 mM PMSF, 5 mM benzamidine). This homogenate was centrifuged at 1,400 g in a Sorvall SS-34 rotor at 4°C for 20 min, and the supernatant was centrifuged at 12,000 g in the same rotor, at 4°C for 20 min. The supernatant was again separated and centrifuged at 100,000 g in a Beckman 60 Ti rotor, at 4°C for 1 h. The pellet was resuspended in 20 ml of the same buffer and subjected to another centrifugation under the same conditions. The final pellet was resuspended in 2–4 ml of buffer, separated into small aliquots, frozen in liquid nitrogen and stored at –80°C until use.

2.5. Protein determination

The protein concentration of purified lipophorins and midgut membrane preparations were determined according to Lowry et al. (1951), in the presence of 0.5% SDS, using bovine serum albumin as standard.

2.6. Binding assay

The midgut membrane preparation was incubated at room temperature (25°C) in the presence of ¹²⁵I-HDLp-L (30 µg/ml, unless otherwise stated) in Binding Buffer (5 mM Tris, 10 mM MOPS, 0.15 M NaCl, 2 mM CaCl₂, 0.25% bovine serum albumin, pH 6.5). The incubation volume was 300 µl and, at the desired times, 200 µl aliquots were removed and applied to 25 mm, type GVWP, pore size 0.22 µm filters (Millipore Corporation, Bedford, MA). The unbound lipophorin was removed by filtration and each filter was washed seven times with 1 ml of ice-cold Binding Buffer. Radioactivity associated with the filters was determined by scintillation counting. For each experiment a blank that did not contain the membrane preparation was carried through the procedure. The radioactivity obtained in the blanks was subtracted from the corresponding experimental samples. For the determination of the non-specific binding, an excess of non-radioactive HDLp-L (2 mg/ml) was added. The specific binding was calculated by subtracting the non-specific binding from the total binding.

2.7. Effect of pH

For measuring the binding of ¹²⁵I-HDLp-L to the membrane preparation at different pH values, 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES), 10 mM

MOPS, 5 mM Tris, 0.15 M NaCl, 0.25% bovine serum albumin, 2 mM CaCl₂ was used for all conditions. The pH was adjusted by the addition of HCl or NaOH.

2.8. Calcium requirement

The requirement for calcium was tested using a modified Binding Buffer containing 2 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetracetic acid (EGTA). CaCl₂ was added in order to obtain the desired Ca²⁺ concentrations, using a computer program (Fabiato and Fabiato, 1979). The pH was adjusted to 6.5.

2.9. Effect of ionic strength

For the determination of the effect of ionic strength on binding, a modified Binding Buffer containing 50 mM NaCl was prepared. Binding Buffers containing different concentrations of NaCl or choline chloride (pH 6.5) were added, in order to obtain the desired ionic strength.

3. Results

When the midgut membrane preparation was incubated with ¹²⁵I-HDLp-L, both specific and non-specific binding of lipophorin were observed (Fig. 1). The time-course of the binding process showed that equilibrium was achieved after about 60 min, when the ¹²⁵I-HDLp-L concentration was 30 µg/ml (Fig. 1A). At a very low ¹²⁵I-HDLp-L concentration (4 µg/ml) a longer time was required to reach equilibrium (Fig. 1B). In most experiments, the ¹²⁵I-HDLp-L concentration used was 30 µg/ml, and the incubations lasted 90 min. Lipophorin binding increased in the presence of increasing amounts of membrane protein in the incubation medium (Fig. 2). For all other experiments 20 µg of membrane protein/ml was used. In a previous paper we used a different binding assay in which the solubilized receptor was incorporated in phospholipid vesicles before assaying (Tsuchida and Wells, 1990). This assay was necessary because we were purifying the receptor. In the present study, we chose to use the membrane preparation to characterize lipophorin binding because we were not purifying the receptor. Preliminary data (not shown) showed that the two assays were comparable.

In order to test whether the iodination reaction affected the binding affinity of lipophorin, HDLp-L was iodinated with nonradioactive iodine, using exactly the same procedure that was used for its labeling with ¹²⁵I. As can be seen in Fig. 3, both the nonradioactively iodinated HDLp-L and the control HDLp-L were equally able to inhibit the binding of the ¹²⁵I-HDLp-L to the membranes. This result showed that the lipophorin labeling procedure did not affect its binding affinity.

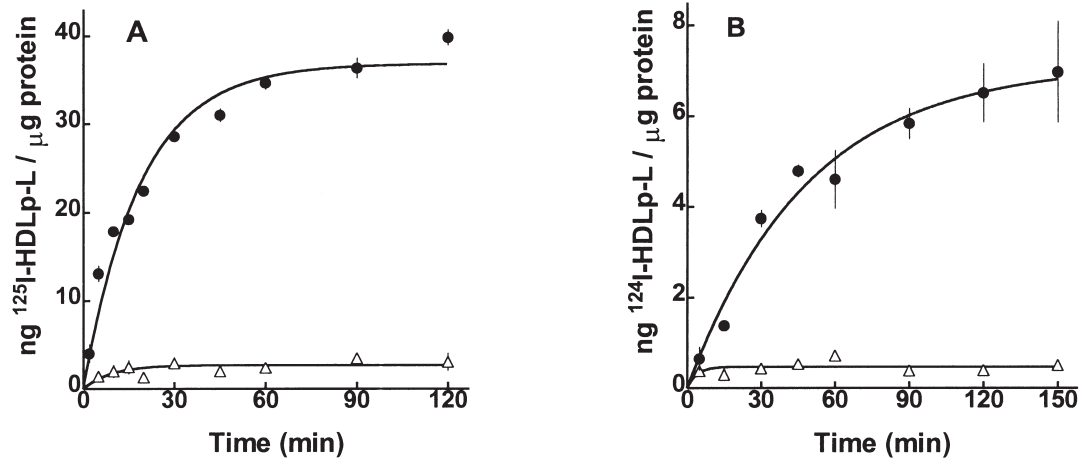


Fig. 1. Time-course of lipophorin binding to midgut membranes. ^{125}I -HDLp-L, at two different concentrations, 30 $\mu\text{g}/\text{ml}$ (A) or 4 $\mu\text{g}/\text{ml}$ (B) was incubated for different times with the midgut membrane preparation (20 μg protein/ml). The specific (●) and non-specific (Δ , in the presence of 2 mg/ml non-radioactive HDLp-L) binding of ^{125}I -HDLp-L to the membranes was determined. The results are expressed as ng bound ^{125}I -HDLp-L/ μg of membrane protein. The vertical bars represent the S.E.M. for four determinations, and where they are not seen they are smaller than the symbols. Other experimental conditions were as described in Materials and methods.

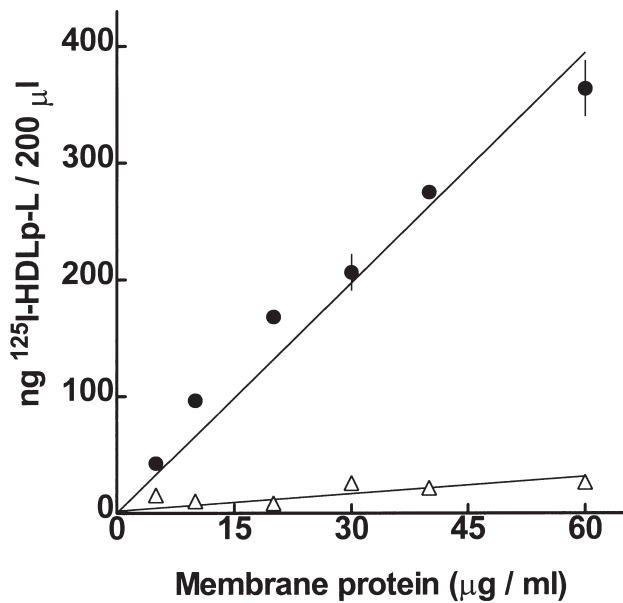


Fig. 2. Effect of the membrane protein concentration on the binding of lipophorin. ^{125}I -HDLp-L (30 $\mu\text{g}/\text{ml}$) was incubated for 90 min with increasing concentrations of midgut membrane protein, and the specific (●) and non-specific (Δ , in the presence of 2 mg/ml nonradioactive HDLp-L) binding of ^{125}I -HDLp-L to the membranes were determined. The results are expressed as ng bound ^{125}I -HDLp-L/200 μl of incubation medium. The vertical bars represent the S.E.M. for four determinations, and where they are not seen they are smaller than the symbols. The lines were calculated using a linear regression analysis. Other experimental conditions were as described in Materials and methods.

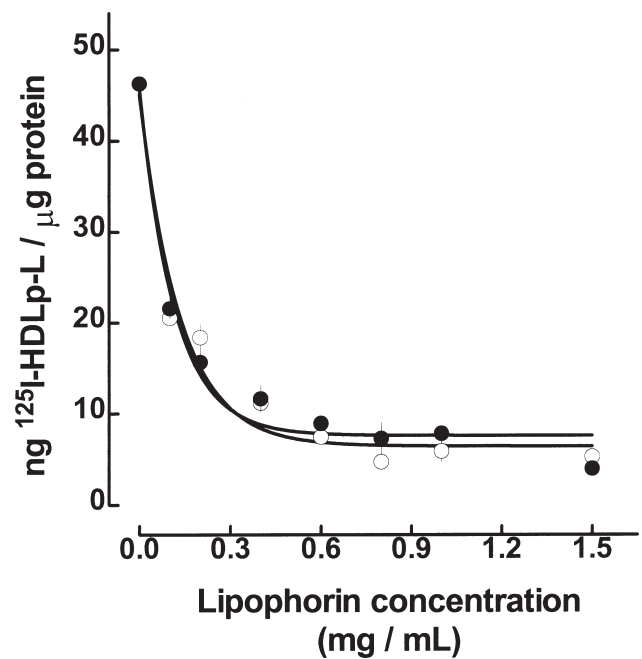


Fig. 3. Analysis of lipophorin affinity for midgut membranes after the iodination reaction. The midgut membrane preparation (20 μg protein/ml) was incubated with ^{125}I -HDLp-L (30 $\mu\text{g}/\text{ml}$) in the presence of increasing concentrations of HDLp-L iodinated with non-radioactive NaI (●), or control HDLp-L (○). After 90 min, the binding of ^{125}I -HDLp-L to the membrane preparation was determined. The results are expressed as ng bound ^{125}I -HDLp-L/ μg of membrane protein. The vertical bars represent the S.E.M. for four determinations, and where they are not seen they are smaller than the symbols. Other experimental conditions were as described in Materials and methods.

When binding was measured with increasing concentrations of ^{125}I -HDLp-L, saturation of the specific binding of lipophorin was observed (Fig. 4A). In this experiment, the incubation time was 3 h to assure that equilibrium was achieved for all lipophorin concentrations used. The specific binding data shown in Fig.

4A were subjected to Scatchard analysis (Scatchard, 1949) (Fig. 4B), which suggested a single type of binding site for lipophorin in the midgut of *M. sexta* larvae. The K_d obtained for six experiments was $1.5 \pm 0.2 \times 10^{-7}$

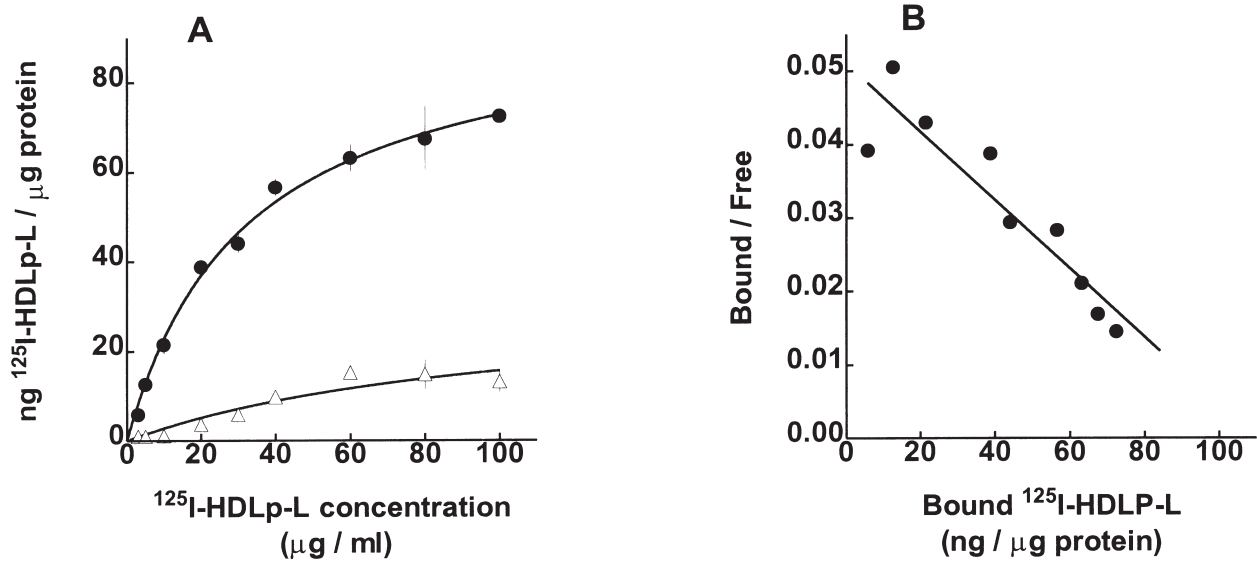


Fig. 4. Determination of lipophorin affinity for midgut membranes. (A) The midgut membrane preparation (20 $\mu\text{g/ml}$) was incubated for 3 h with increasing concentrations of ^{125}I -HDLp-L. The specific (\bullet) and non-specific (Δ , in the presence of 2 mg/ml non-radioactive HDLp-L) binding of ^{125}I -HDLp-L to the membranes were determined. The results are expressed as ng bound ^{125}I -HDLp-L/ μg of membrane protein. The vertical bars represent the S.E.M. for four determinations, and where they are not seen they are smaller than the symbols. Other experimental conditions were as described in Materials and methods. (B) Scatchard plot of the specific binding data from panel (A). A K_d of $1.5 \pm 0.2 \times 10^{-7}$ M was calculated from six experiments.

M, with a maximal binding capacity of 127 ± 17 ng/ μg of membrane protein.

The specific binding of ^{125}I -HDLp-L to the midgut membranes showed a maximum around pH 5.5 (Fig. 5). The blanks, in the absence of membranes, showed that no precipitation of lipophorin was observed in the pH range that was tested (data not shown). All other experiments were done at pH 6.5 because this was the pH we measured for hemolymph of day 4 feeding fifth instar larvae. The same result for the pH optimum was obtained if the assay was performed using other buffer combinations, such as N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)/Tris or bis-(2-hydroxyethyl) iminotris (hydroxymethyl) methane (Bis-Tris)/MOPS/Tris (data not shown).

Although the binding of many ligands to their receptors has been shown to depend on calcium (George et al., 1987; Schneider et al., 1982; Tsuchida and Wells, 1990), the addition of 2 mM EGTA in the absence of CaCl_2 did not inhibit lipophorin binding to the midgut membranes (Table 1). Also, no difference was observed when other divalent cations, such as magnesium or strontium, were added (data not shown).

Suramin, a polysulfated polycyclic hydrocarbon known to inhibit the binding of lipoproteins to their receptors (Schneider et al., 1982; Röhrkasten and Ferenz, 1987; Tsuchida and Wells, 1990; Dhadialla et al., 1992) also interfered with lipophorin binding to the midgut membranes, and binding was completely abolished at a concentration of 2 mM suramin (Fig. 6A). In this experiment, only total binding was determined, because in the

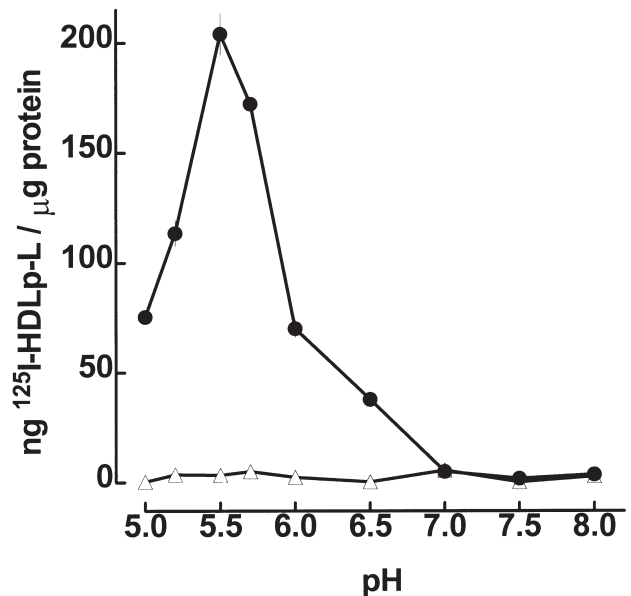


Fig. 5. Effect of pH on the binding of lipophorin to midgut membranes. ^{125}I -HDLp-L (30 $\mu\text{g/ml}$) was incubated for 90 min with the midgut membrane preparation (20 μg protein/ml) at different pH values, in a buffer containing: 10 mM MES, 10 mM MOPS, 5 mM Tris, 0.15 M NaCl, 0.25% bovine serum albumin and 2 mM CaCl_2 . The pH was adjusted by the addition of HCl or NaOH. The specific (\bullet) and non-specific (Δ , in the presence of 2 mg/ml nonradioactive HDLp-L) binding were determined. The results are expressed as ng bound ^{125}I -HDLp-L/ μg of membrane protein. The vertical bars represent the S.E.M. for four determinations, and where they are not seen they are smaller than the symbols. Other experimental conditions were as described in Materials and methods.

Table 1
Effect of calcium on the binding of lipophorin to the midgut membrane preparation^a

Ca ²⁺ (mM)	(ng ¹²⁵ I-HDLp-L/μg protein)	
	Specific binding	Non-specific binding
0	50.8±1.1	11.7±0.3
0.5	43.8±2.2	9.7±0.2
1.0	46.5±1.6	8.9±0.4
2.0	41.1±1.7	10.8±0.6

^a ¹²⁵I-HDLp-L (30 μg/ml) was incubated with the midgut membrane preparation (20 μg protein/ml) in the presence of 2 mM EGTA and the required CaCl₂ concentrations to obtain 0; 0.5; 1.0 and 2.0 mM Ca²⁺. The specific and non-specific (in the presence of 2 mg/ml non-radioactive HDLp-L) bindings were determined. The results are expressed as ng bound ¹²⁵I-HDLp-L/μg of membrane protein, and they are means and S.E.M. for four determinations. Other conditions were as described in Materials and methods.

presence of the excess nonradioactive lipophorin (2 mg/ml) used to determine nonspecific binding, the addition of suramin at concentrations higher than 0.2 mM caused lipophorin precipitation. This precipitation did not occur if only ¹²⁵I-HDLp-L (30 μg/ml) was used. If 2 mM suramin were added after ¹²⁵I-HDLp-L had been allowed to bind to the membranes, not all the bound lipoprotein could be displaced, and no difference was observed if the drug was added to the medium at different times after the beginning of the incubation (Fig. 6B).

Lipophorin binding to the midgut membranes was strongly affected by the ionic strength, and was com-

pletely abolished when the salt concentration (NaCl or choline chloride) was above 200 mM (Fig. 7). At 150 mM, the same binding level was observed in the presence of NaCl, KCl, LiCl and choline chloride (data not shown).

The affinities of the midgut membranes for lipophorins with different densities were compared by their ability to affect the binding of ¹²⁵I-HDLp-L. We used increasing concentrations of nonradioactive HDLp-L (density 1.138 g/ml); HDLp-W₂ (density 1.177 g/ml; Prasad et al., 1986); VHDLp-E (density 1.238 g/ml; Kawooya et al., 1988); or HDLp-L from insects raised on a high-fat diet (density 1.115 g/ml; Fernando-Warnakulasuriya et al., 1988). As can be seen in Fig. 8, these different lipophorins did not show any significant difference in their affinity for the midgut membranes.

4. Discussion

We have characterized the binding of lipophorin to a midgut membrane preparation from larval *M. sexta*. The assay measured the binding of ¹²⁵I-HDLp-L, which was shown to be appropriate for the characterization of the HDLp-L binding because iodination did not affect the affinity of HDLp for the membranes. This was an important control as there is always the possibility that a non-metabolically labeled ligand might have an altered affinity. The midgut membrane showed a single type of high affinity binding site with a K_d=1.5±0.2×10⁻⁷ M (~48 μg/ml). As the lipophorin concentration in the feed-

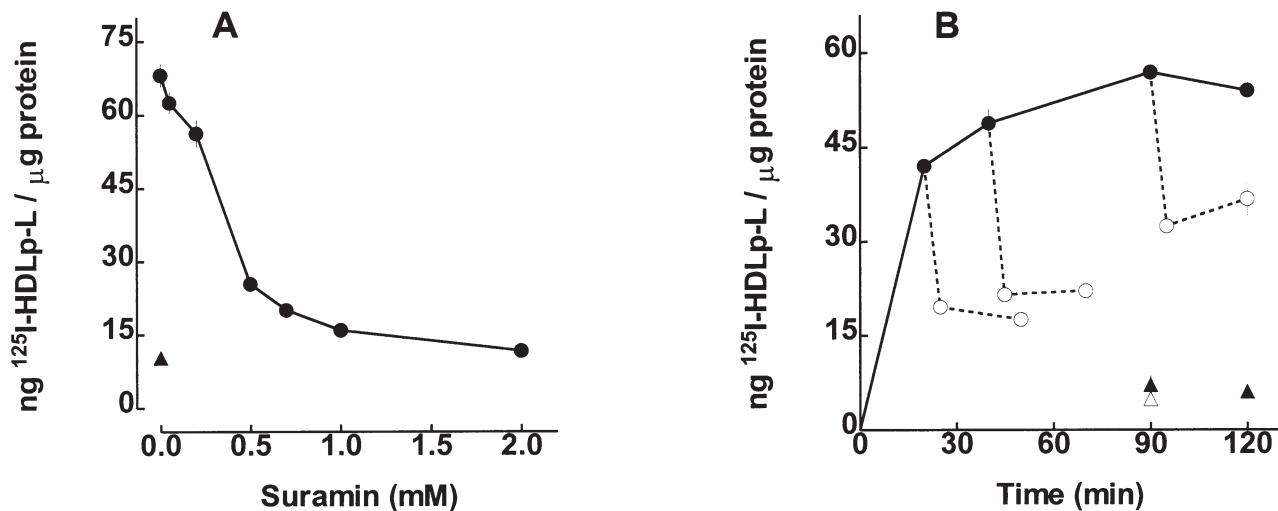


Fig. 6. The effect of suramin on the binding of lipophorin to midgut membranes. (A) The total binding of ¹²⁵I-HDLp-L (30 μg/ml) to the midgut membrane preparation (20 μg protein/ml) was determined in the presence of increasing concentrations of suramin (●). The non-specific binding (▲) was measured in the presence of nonradioactive HDLp-L (2 mg/ml), in the absence of suramin. (B) ¹²⁵I-HDLp-L (30 μg/ml) was incubated with the midgut membrane preparation (20 μg protein/ml) and after 20, 40 or 90 min, suramin (2 mM) was added. After an additional 5 and 30 min, the total binding of the ¹²⁵I-HDLp-L to the membrane was determined (○). In the absence of suramin the total (●) and non-specific (▲, in the presence of 2 mg/ml non-radioactive HDLp-L) binding were determined. In (△) suramin was present from the beginning of the incubation. The results are expressed as ng bound ¹²⁵I-HDLp-L/μg of membrane protein. The vertical bars represent the S.E.M. for four determinations, and where they are not seen they are smaller than the symbols. Other experimental conditions were as described in Materials and methods.

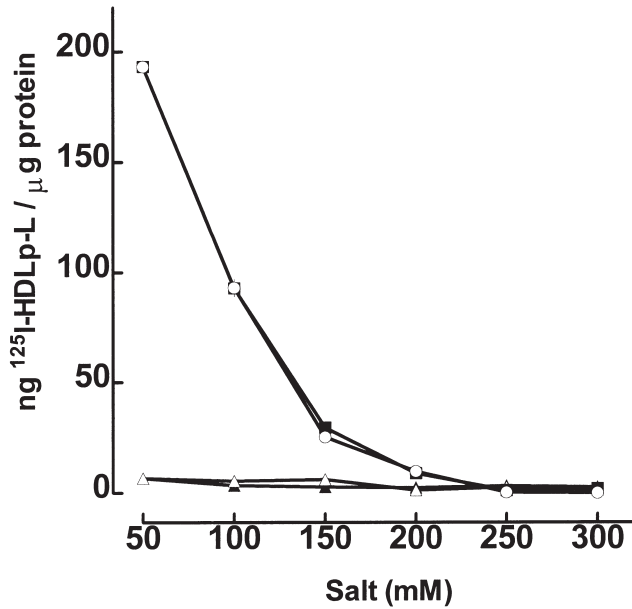


Fig. 7. The effect of ionic strength on lipophorin binding to midgut membranes. ^{125}I -HDLp-L (30 $\mu\text{g}/\text{ml}$) was incubated for 90 min with the midgut membrane preparation (20 μg protein/ml) in the presence of increasing concentrations of NaCl or choline chloride. The specific binding (NaCl, \circ ; choline chloride, \blacksquare), and the non-specific binding, in the presence of 2 mg/ml nonradioactive HDLp-L (NaCl, \triangle ; choline chloride, \blacktriangle), were determined. The results are expressed as ng bound ^{125}I -HDLp-L/ μg of membrane protein. The vertical bars represent the S.E.M. for four determinations. Other experimental conditions were as described in Materials and methods.

ing fifth instar *M. sexta* hemolymph is much higher than this, 4–5 mg/ml (Prasad et al., 1987), it is probable that the midgut binding sites are always saturated, which may contribute to a high efficiency of lipid transfer. The fact that the hemolymph concentration of lipophorin is much higher than the K_d for lipophorin binding seems to be a common pattern in insects, as the same kind of results were described for the fat body of *M. sexta* and *L. migratoria* (Tsuchida and Wells, 1990; Schulz et al., 1991) and for the fat body and ovaries of *Rhodnius prolixus* (Atella et al., 1992; Machado et al., 1996).

Lipophorin binding to the midgut was very sensitive to pH, and was optimal around pH 5.5, which is below the pH of the hemolymph (~6.5). Although we do not know the significance of this pH-dependence, the result is consistent with the one reported for *L. migratoria* fat body (Schulz et al., 1991). A different pH dependence was described for the lipophorin receptor from the fat body of *M. sexta* (Tsuchida and Wells, 1990), which showed an optimum at pH 6.5, but in that study a solubilized receptor preparation was used.

Other receptors, like the bovine adrenal cortex receptor for low-density lipoprotein (LDL) and the chicken oocyte receptor for LDL and very low-density lipoprotein (VLDL) require calcium for the interaction with the lipoproteins (Schneider et al., 1982; George et al., 1987). However, the binding of lipophorin to the *M. sexta*

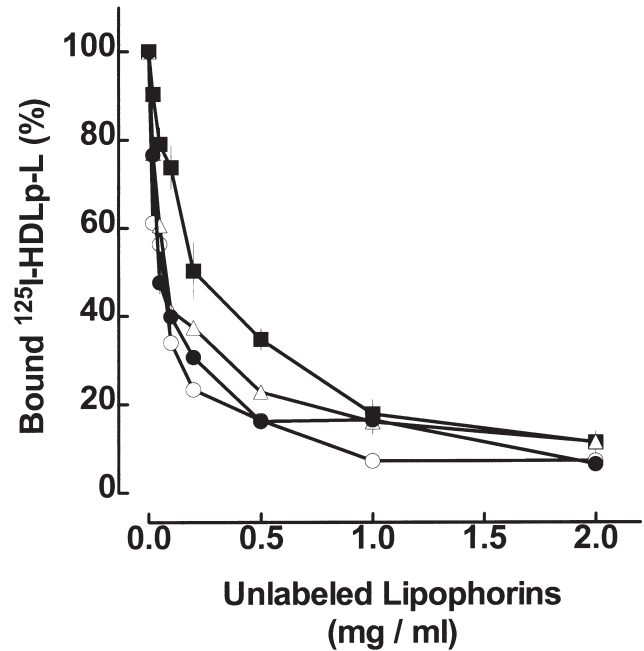


Fig. 8. ^{125}I -HDLp-L (30 $\mu\text{g}/\text{ml}$) was incubated for 90 min with the midgut membrane preparation (20 μg protein/ml) in the absence or presence of increasing concentrations of non-radioactive HDLp-L (\bullet), HDLp-W₂ (\triangle), HDLp-L from insects raised in a high-fat diet (\circ), or VHDLp-E (\blacksquare). The results from two independent experiments were normalized and are expressed as percentage of the binding found in the absence of non-radioactive lipophorins. The vertical bars represent the S.E.M. for eight determinations, and where they are not seen they are smaller than the symbols. Other experimental conditions were as described in Materials and methods.

midgut did not show a requirement for calcium. This result was different from that obtained with the fat body receptor (Tsuchida and Wells, 1990). Both the Ca^{2+} and pH results suggest that the lipophorin receptors in midgut and fat body have different properties, but a detailed description of the differences will depend on obtaining a purified midgut receptor.

Lipophorin binding to the midgut membrane preparation was inhibited by the presence of suramin, as was described for the fat body receptor (Tsuchida and Wells, 1990) and for other insect and vertebrate receptors (Schneider et al., 1982; Dhadialla et al., 1992). On the other hand, bound lipophorin could not be completely displaced by the addition of suramin to the medium, which was similar to the binding of vitellogenin to its receptor from *L. migratoria* oocytes (Röhrkasten and Ferenz, 1987). The fact that lipophorin at a high concentration (2 mg/ml) precipitated when suramin was added suggests that the observed suramin inhibition of the binding was due to its interaction with lipophorin, rather than with the membrane. At the low lipophorin concentration (30 $\mu\text{g}/\text{ml}$) used for the determination of the total binding, suramin interaction with lipophorin might occur, which could lead to the inhibition of the binding, but not to lipoprotein precipitation. In *L. migratoria*

(Schulz et al., 1991), the binding of lipophorin to a fat body protein extract was inhibited by heparin, also a polysulfated substance. The effect was not observed when the fat body extract was only preincubated with heparin, suggesting that the inhibition resulted from the interaction of heparin with lipophorin. It is possible that these two substances, suramin and heparin, interact with lipoproteins in a similar way.

The complete inhibition of lipophorin specific binding at high ionic strength indicated the importance of electrostatic interactions for the binding of the lipoprotein to the midgut. A similar effect was also observed for the binding of lipophorin to the fat body from *L. migratoria* (Dantuma et al., 1996) and for the binding of LDL, but not high-density lipoprotein (HDL), to receptors in the rat corpus luteum (Hwang and Jairam Menon, 1983). Although the sodium concentration is very low in the hemolymph of *M. sexta* and other lepidopterans, potassium and magnesium are present in relatively high concentrations (Jungreis et al., 1973; Mullins, 1985). The buffer we used for the binding assays contained 0.15 M NaCl, but the same binding levels are obtained in the presence of choline chloride (Fig. 7), KCl, LiCl, or MgCl₂ (data not shown).

Lipophorins of different densities and lipid contents did not show any significant difference in affinity for the midgut membrane. This result was different from that obtained with the *M. sexta* fat body receptor, which showed higher affinities for lipophorins containing more lipids (Tsuchida and Wells, 1990). In feeding larvae, lipophorin is loaded with lipids at the midgut and then it transports them to the fat body (Tsuchida and Wells, 1988), and it might have been expected that the denser, lipid-poor, particles would have a higher affinity for the midgut. A hypothesis for the regulation of lipid transport from midgut to fat body was based of this supposition (Soulages and Wells, 1994). However, the data presented in Fig. 8 showed that the midgut membrane does not show a lipid content-dependent affinity for lipophorins. This means that the above hypothesis is not correct and other possibilities for regulation of lipid transport between midgut and fat body in larval *M. sexta* need to be investigated.

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References

Atella, G.C., Gondim, K.C., Masuda, H., 1992. Transfer of phospholipids from the fat body to lipophorin in *Rhodnius prolixus*. *Arch. Insect Biochem. Physiol.* 19, 133–144.

Atella, G.C., Gondim, K.C., Masuda, H., 1995. Loading of lipophorin particle with phospholipids at the midgut of *Rhodnius prolixus*. *Arch. Insect Biochem. Physiol.* 30, 337–350.

Bauerfeind, R., Komnick, H., 1992. Lipid-loading and unloading of lipophorin in the midgut epithelium of dragonfly larvae *Aeshna cyanea*. *J. Insect Physiol.* 38, 147–160.

Beenackers, A.M.Th., Chino, H., Law, J.H., 1988. Lipophorin nomenclature. *Insect Biochem.* 18, 1–2.

Blacklock, B.J., Ryan, R.O., 1994. Hemolymph lipid transport. *Insect Biochem. Molec. Biol.* 24, 855–873.

Chino, H., 1985. Lipid transport: biochemistry of hemolymph lipophorin. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 10. Pergamon Press, Oxford, pp. 115–134.

Chino, H., Downer, R.G.H., Wyatt, G.R., Gilbert, L.I., 1981. Lipophorins, a major class of lipoproteins of insect haemolymph. *Insect Biochem.* 11, 491.

Dantuma, N.P., Van Marrewijk, W.J.A., Wynne, H.J., Van der Horst, D.J., 1996. Interaction of an insect lipoprotein with its binding site at the fat body. *J. Lipid Res.* 37, 1345–1355.

Dantuma, N.P., Pijnenburg, M.A.P., Diederer, J.H.B., Van der Horst, D.J., 1997. Developmental down-regulation of receptor-mediated endocytosis of an insect lipoprotein. *J. Lipid Res.* 38, 254–265.

Dantuma, N.P., Pijnenburg, M.A.P., Diederer, J.H.B., Van der Horst, D.J., 1998. Electron microscopic visualization of receptor-mediated endocytosis of Dil-labeled lipoproteins by diaminobenzidine photo-conversion. *J. Histochem. Cytochem.* 46, 1085–1089.

Dantuma, N.P., Potters, M., De Winther, M.P.J., Tensen, C.P., Kooiman, F.P., Bogerd, J., Van der Horst, D.J., 1999. An insect homolog of the vertebrate very low density lipoprotein receptor mediates endocytosis of lipophorins. *J. Lipid Res.* 40, 973–978.

Dhadialla, T.S., Hays, A.R., Raikhel, A.S., 1992. Characterization of the solubilized mosquito vitellogenin receptor. *Insect Biochem. Molec. Biol.* 22, 803–816.

Downer, R.G.H., Chino, H., 1985. Turnover of protein and diacylglycerol components of lipophorin in insect haemolymph. *Insect Biochem.* 15, 627–630.

Fabiato, A., Fabiato, F., 1979. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol., Paris* 75, 463–505.

Fernando-Warnakulasuriya, G.J.P., Tsuchida, K., Wells, M.A., 1988. Effect of dietary lipid content on lipid transport and storage during larval development of *Manduca sexta*. *Insect Biochem.* 18, 211–214.

George, R., Barber, D.L., Schneider, W.J., 1987. Characterization of the chicken oocyte receptor for low and very low density lipoproteins. *J. Biol. Chem.* 262, 16838–16847.

Hwang, J., Jairam Menon, K.M., 1983. Characterization of low density and high density lipoprotein receptors in the rat corpus luteum and regulation by gonadotropin. *J. Biol. Chem.* 258, 8020–8027.

Jungreis, A.M., Jatlow, P., Wyatt, G.R., 1973. Inorganic ion composition of haemolymph of the cecropia silkworm: changes with diet and ontogeny. *J. Insect Physiol.* 19, 225–233.

Kanost, M.R., Kawooya, J.K., Law, J.H., Ryan, R.O., Van Heusden, M.C., Ziegler, R., 1990. Insect hemolymph proteins. *Adv. Insect Physiol.* 22, 299–396.

Kawooya, J.K., Osir, E.O., Law, J.H., 1988. Uptake of the major hemolymph lipoprotein and its transformation in the insect egg. *J. Biol. Chem.* 263, 8740–8747.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature, London* 227, 680–685.

Lowry, O.H., Rosebrough, N.J., Farr, A.R., Randall, R.J., 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.

Machado, E.A., Atella, G.C., Gondim, K.C., De Souza, W., Masuda,

- H., 1996. Characterization and immunocytochemical localization of lipophorin binding sites in the oocytes of *Rhodnius prolixus*. *Arch. Insect Biochem. Physiol.* 31, 185–196.
- Mullins, D.E., 1985. Chemistry and physiology of the hemolymph. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 3. Pergamon Press, Oxford, pp. 355–400.
- Prasad, S.V., Ryan, R.O., Law, J.H., Wells, M.A., 1986. Changes in lipoprotein composition during larval-pupal metamorphosis of an insect *Manduca sexta*. *J. Biol. Chem.* 261, 558–562.
- Prasad, S.V., Tsuchida, K., Cole, K.D., Wells, M.A., 1987. Lipophorin biosynthesis during the life cycle of the tobacco hornworm, *Manduca sexta*. In: Law, J.H. (Ed.), *Molecular Entomology: ICN-UCLA Symposium on Molecular and Cellular Biology*, vol. 49. Academic Press, Orlando, Florida, pp. 267–273.
- Röhrkasten, A., Ferenz, H.J., 1987. Inhibition of yolk formation in locust oocytes by trypan blue and suramin. *Roux Arch. Dev. Biol.* 196, 165–168.
- Scatchard, G., 1949. The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* 51, 660–672.
- Schneider, W.J., Beisiegel, U., Goldstein, J.L., Brown, M.S., 1982. Purification of the low density lipoprotein receptor, an acidic glycoprotein of 164,000 molecular weight. *J. Biol. Chem.* 257, 2664–2673.
- Schulz, T.K.F., Van der Horst, D.J., Beenackers, A.M.TH., 1991. Binding of locust high-density lipophorin to fat body proteins monitored by an enzyme-linked immunosorbant assay. *Biol. Chem. Hoppe-Seyler* 372, 5–12.
- Shapiro, J.P., Law, J.H., Wells, M.A., 1988. Lipid transport in insects. *A. Rev. Entomol.* 33, 297–318.
- Soulages, J.L., Wells, M.A., 1994. Lipophorin: the structure of an insect lipoprotein and its role in lipid transport in insects. *Adv. Protein Chem.* 45, 371–415.
- Surholt, B., Van Doorn, J.M., Goldberg, J., Van der Horst, D.J., 1992. Insect lipophorin interconversions: compositional analysis of high- and low-density lipophorin of *Acherontia atropos* and *Locusta migratoria*. *Biol. Chem. Hoppe-Seyler* 373, 13–20.
- Tsuchida, K., Wells, M.A., 1988. Changes in lipophorin composition during the fourth to fifth instar molt of *Manduca sexta*. *Insect Biochem.* 18, 263–268.
- Tsuchida, K., Wells, M.A., 1990. Isolation and characterization of a lipoprotein receptor from the fat body of an insect *Manduca sexta*. *J. Biol. Chem.* 265, 5761–5767.
- Van Antwerpen, R., Linnemans, W.A.M., Van der Horst, D.J., Beenackers, A.M.TH., 1988. Immunocytochemical localization of lipophorins in the flight muscles of the migratory locust (*Locusta migratoria*) at rest and during flight. *Cell Tiss. Res.* 252, 661–668.
- Van Antwerpen, R., Wynne, H.J.A., Van der Horst, D.J., Beenackers, A.M.TH., 1989. Binding of lipophorin to the fat body of the migratory locust. *Insect Biochem.* 19, 809–814.
- Van der Horst, D.J., 1990. Lipid transport function of lipoproteins in flying insects. *Biochim. Biophys. Acta* 1047, 195–211.
- Van Heusden, M.C., Van der Horst, D.J., Voshol, J., Beenackers, A.M.TH., 1987. The recycling of protein components of the flight-specific lipophorin in *Locusta migratoria*. *Insect Biochem.* 17, 771–776.
- Ziegler, R., Willingham, L.A., Sanders, S.J., Tamen-Smith, L., Tsuchida, K., 1995. Apolipophorin-III and the adipokinetic hormone in lipid metabolism of larval *Manduca sexta*. *Insect Biochem. Molec. Biol.* 25, 101–108.