Biosynthesis and secretion of insect lipoprotein

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Abstract Biosynthesis of high density lipophorin (HDLp) was studied in larvae and adults of the migratory locust, *Locusta migratoria*. In an in vitro system, fat bodies were incubated in a medium containing a mixture of tritiated amino acids. Using SDS-PAGE and immunoblotting, it was shown that larval and adult fat bodies secreted both HDLp apoproteins, apolipophorin I (apoLp-I) and apolipophorin II (apoLp-II). Radiolabel was recovered in both apoproteins, indicative of de novo synthesis. The density of the fractions containing the apoproteins synthesized and secreted by larval and adult fat bodies was determined by density gradient ultracentrifugation. A radiolabeled protein fraction was found at density 1.12 g/ml. Using an enzyme-linked immunosorbent assay for detecting apoLp-I and apoLp-II, it was demonstrated that both apoproteins were present in this fraction, which had a density identical to that of circulating HDLp in hemolymph. Lipid analysis revealed that it contained phospholipid, diacylglycerol, sterol, and hydrocarbons. From these results it is concluded that the fat body of the locust synthesizes both apoLp-I and apoLp-II, which are combined with lipids to a lipoprotein particle that is released into the medium as HDLp.—**Weers, P. M. M., D. J. Van der Horst, W. J. A. Van Marrewijk, M. Van den Eijnden, J. M. Van Doorn and A. M. T. Beenakkers.** Biosynthesis and secretion of insect lipoprotein. *J. Lipid Res.* 1992. **33:** 485–491.

Supplementary key words locust • high density lipophorin • apolipophorin • fat body • enzyme-linked immunosorbent assay

High density lipophorin (HDLp) is the major lipid transporting vehicle in insect blood (hemolymph) (1). In larvae and resting adults, HDLp transports various classes of lipids between the midgut (site of absorption), fat body (storage organ), and peripheral tissues. During lipid delivery, the basic matrix of the HDLp particle is not affected and can be reused (for recent reviews see 2–5). Basically, HDLp is composed of two distinct apoproteins: one molecule of apolipophorin I (apoLp-I, molecular mass ca. 240 kDa) and one molecule of apolipophorin II (apoLp-II, molecular mass ca. 80 kDa); both apolipophorins are glycosylated (6). The lipid components of HDLp comprise phospholipid, diacylglycerol (DAG), sterol, and hydrocarbons (7).

During insect flight, HDLp is converted into low density lipophorin (LDLp) by uptake of large amounts of DAG that is mobilized from the fat body by the action of adipokinetic hormone, and this particle is associated with several molecules of a third apolipophorin (apoLp-III, molecular mass ca. 18–20 kDa) (8–11). Lipid hydrolysis at the flight muscles results in dissociation of LDLp into HDLp and apoLp-III in hemolymph, thus LDLp acts as a reusable shuttle mechanism for supplying large amounts of DAG as an energy source for the flight muscles during insect flight (12).

HDLp biosynthesis has been studied particularly in the larvae of the holometabolous insects *Manduca sexta* (13) and *Diatraea grandiosella* (14), using the fat body as the site of lipophorin synthesis. The data reported concerning the nature of the released lipophorin were, however, different. In this paper we describe the biosynthesis and release of lipophorin in both larvae and adults of the hemimetabolous migratory locust, *Locusta migratoria*. We provide evidence that the apoproteins are synthesized in the fat body and subsequently released as HDLp. Based on the results presented in this paper we propose a new model for HDLp biosynthesis.

MATERIALS AND METHODS

Animals

Locusta migratoria migratorioides were reared under crowded conditions as previously described (15). Male larval (5th instar, 5 days after the last larval ecdysis) or adult (8 days after imaginal ecdysis) locusts were used.

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; apoLp, apolipophorin; HDLp, high density lipophorin; LDLp, low density lipophorin; DAG, diacylglycerol; MAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

In vitro fat body incubations

Fat bodies of larval or adult locusts were dissected and rinsed three times in saline (10 mM HEPES, 150 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.0). Incubations were performed in 250 µl saline containing 100 units/ml penicillin and 0.1 mg/ml streptomycin, in a shaking water bath at 32°C. For radiolabeling experiments, 10 µCi of a tritiated amino acid mixture (Amersham International, sp act ~ 45 Ci/mmol) was added.

In some experiments 15 µg/ml tunicamycin (Boehringer Mannheim) was included in the incubation medium.

Incorporation of radiolabel into proteins

The time course of incorporation of tritiated amino acids into proteins synthesized and secreted by the fat body was established by the filter disk method of Mans and Novelli (16). At the times given during fat body incubation, aliquots of 25 µl medium were taken and spotted on filter disks (Millipore); these were washed with 10% trichloroacetic acid and dissolved in Filter Count (Packard). Radioactivity was measured using a liquid scintillation spectrometer (Packard, model 4550).

Immunoprecipitation

Medium from fat body incubations was subjected to immunoprecipitation. Medium was incubated with rabbit anti-apoLp-I or apoLp-II serum (17) and incubated for 3 h at 37°C, followed by addition of 0.7 mg of goat anti-rabbit Immunobead reagent (Bio-Rad) and bovine serum albumin, whereafter the incubation was continued overnight at 4°C. The immunoprecipitate was washed three times with saline, then incubated for 20 min at 60°C in saline containing 2% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol. This SDS-treated fraction was centrifuged and the supernatant was collected and stored at –20°C until use.

Gel electrophoresis and blotting

SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli and Favre (18), essentially as described by Van der Horst et al. (15). Proteins were separated on 7% SDS-PAGE, transferred to nitrocellulose (Schleicher & Schuell), and stained with 0.05% Amido black 10B. The distribution of radiolabel in protein bands was determined by scanning the blots with a linear radioactivity analyzer (Berthold, model LB 2842).

Immunoblotting

Immunoblotting was carried out essentially according to the method of Burnette (19) as described by Schulz et al. (17), using monoclonal antibodies (MAbs) specific for apoLp-I and apoLp-II. The production and characteristics of these MAbs have been described (17).

Density gradient ultracentrifugation

In order to determine the density of the lipoproteins synthesized and secreted by the fat body, the media from eight incubations were pooled and subjected to density gradient ultracentrifugation essentially as described by Shapiro, Keim, and Law (20). After dialysis against saline, KBr was added to the medium (final density 1.3 g/ml). This solution was overlayered with a KBr solution with density 1.05 g/ml. After centrifugation in a vertical rotor (Sorvall, TV 850) for 3.5 h at 49,000 rpm (4°C), the resulting gradient was fractionated and monitored for protein at 280 nm (Uvicord S, LKB). Radioactivity was measured in an Emulsifier Safe scintillator (Packard). The final density gradient was determined gravimetrically and ranged from 1.09 to 1.31 g/ml. In addition, the density of control HDLp was determined by collecting hemolymph of adult locusts followed by density gradient ultracentrifugation (as described above).

Enzyme-linked immunosorbent assay (ELISA) for detection of apoLp-I and apoLp-II

To quantify apoLp-I and apoLp-II secreted during fat body incubations, the sandwich ELISA described by Leroy et al. (21) was modified. In some experiments, medium was first subjected to density gradient ultracentrifugation and the resulting gradient was fractionated and dialyzed against phosphate-buffered saline (PBS). Microplates (96-well PVC plates, Flow Laboratories) were coated overnight at 4°C with rabbit polyclonal apoLp-I and apoLp-II antiserum (20 µg/ml). After washing with water, the wells were incubated with 100-µl samples of fat body incubation medium, and incubated overnight at 4°C. In addition, HDLp samples were used for the preparation of a standard curve in a working range of 5 to 100 ng/ml. After washing with water (3×), PBS containing 0.5% Tween 20 (2×) and water (3×), the wells were incubated with 100 µl of a mixture of MAbs directed against apoLp-I and apoLp-II, diluted 1/2,000 in PBS containing 5% normal goat serum (to reduce nonspecific binding). After incubation for 2 h at 37°C, the wells were washed and incubated for 2 h at 37°C with 100 µl of diluted (1/3,000) goat anti-mouse antibodies coupled to peroxidase (TAGO, Burlingame, CA). The wells were washed with water and bound peroxidase

was visualized with 75 µl of *o*-phenylenediamine substrate (Baker Chemicals). The enzymatic color reaction was stopped by adding 75 µl 2 M H_2SO_4, whereafter the extinction was measured at 492 nm.

Determination of lipid classes

Lipids of in vitro synthesized lipoproteins derived from 50 fat body incubations or HDLp isolated from hemolymph were extracted with chloroform–methanol according to Bligh and Dyer (22). Lipid classes were separated by thin-layer chromatography (TLC) on silica gel G plates (pre-coated TLC plates SIL G-25, Machery-Nagel, Düren, Germany) using the method of Freeman and West (23). Lipids were separated on plates developed in benzene–diethyl–ether–ethanol–acetic acid 50:40:2:0.2 (v/v/v/v), and subsequently in hexane–diethyl ether 94:6 (v/v). Additionally, a separation of the phospholipid classes was performed on silica gel plates (pre-coated TLC plates SIL-60, Machery-Nagel, Düren, Germany), according to Vitiello and Zanetta (24), using methyl acetate–n-propanol–chloroform–methanol–0.25% aqueous KCl 25:25:25:10:9 (v/v/v/v/v) as the solvent system. Individual lipid classes were visualized by spraying gels with 3% cupric(II) acetate in 8% H_3PO_4 and heating at 180°C for 15 min. The separated lipids were scanned with an Ultroscan XL Laser densitometer (Pharmacia) and the relative amount of each lipid class was quantified.

RESULTS

Fat body apoprotein synthesis

The ability of fat bodies from adult locusts and 5th instar larvae to synthesize and secrete proteins into the incubation medium under our experimental conditions was investigated. Incorporation of radiolabeled amino acids into proteins was measured, using a mixture of tritiated amino acids. The results are shown in **Fig. 1.** Incorporation of radiolabel appeared to occur at a constant level for at least 3 h, suggesting functioning of the fat body for the duration of the incubation. On the basis of these results, a time of 3 h was chosen for further fat body incubations.

Secreted apoproteins were precipitated with specific antiserum; the precipitate was subjected to SDS-PAGE followed by blotting on nitrocellulose. The results are shown in **Fig. 2.** On nitrocellulose blots three major protein bands appeared. By immunostaining using MAbs specific for apoLp-I and apoLp-II, two proteins could be identified as apoLp-I and apoLp-II, respectively. ApoLp-I was found to have a molecular mass of ~ 220 kDa. For apoLp-II a molecular mass of ~ 72 kDa was obtained. No differences between larval and adult apoproteins were demonstrable concerning molecular

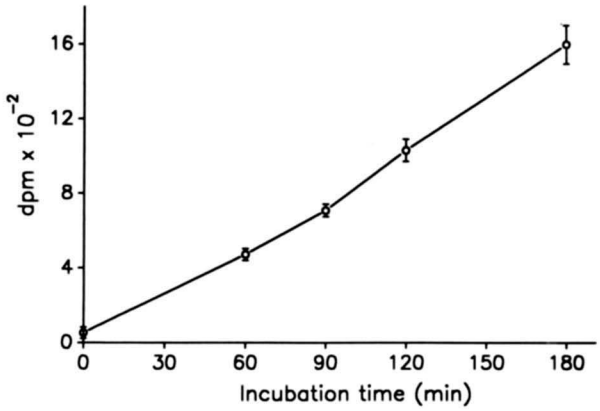

Fig. 1. Time course of radiolabel incorporation into proteins synthesized and secreted by locust fat body. Adult fat bodies were incubated in saline containing tritiated amino acids. At the intervals indicated, aliquots of 25 µl were taken and spotted on filters that were washed with trichloroacetic acid. Filters were dissolved and radioactivity was measured. Each point represents a 25-µl sample (mean ± SD, n = 3).

mass and immunoreactivity. Since we included bovine serum albumin in our purification during the immunoprecipitation to minimize nonspecific interactions, the abundant 67 kDa protein recovered probably represents this added bovine serum albumin.

The proteins recovered on the nitrocellulose blots were analyzed for incorporation of radioactivity. In **Fig. 3** a scan of the radioactivity into these proteins

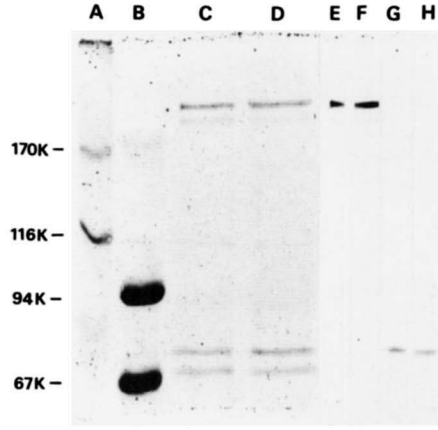

Fig. 2. SDS-PAGE and immunoblotting of in vitro synthesized and secreted proteins. In vitro fat body incubations were followed by immunoprecipitation from the medium with antiserum specific for apoLp-I and apoLp-II. The precipitate was electrophoresed on SDS-PAGE (7%). Proteins on the gel were blotted on nitrocellulose, stained for proteins (lanes A–D), or immunostained with MAbs specific for apoLp-I or apoLp-II (lanes E–H). Lane A: marker proteins (α_2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa); lane B: marker proteins (phosphorylase b, 94 kDa, bovine serum albumin, 67 kDa); lane C: proteins secreted by adult fat bodies; lane D: proteins secreted by larval fat bodies; lane E: adult proteins stained with α-apoLp-I; lane F: larval proteins stained with α-apoLp-I; lane G: adult proteins stained with α-apoLp-II; lane H: larval proteins stained with α-apoLp-II.

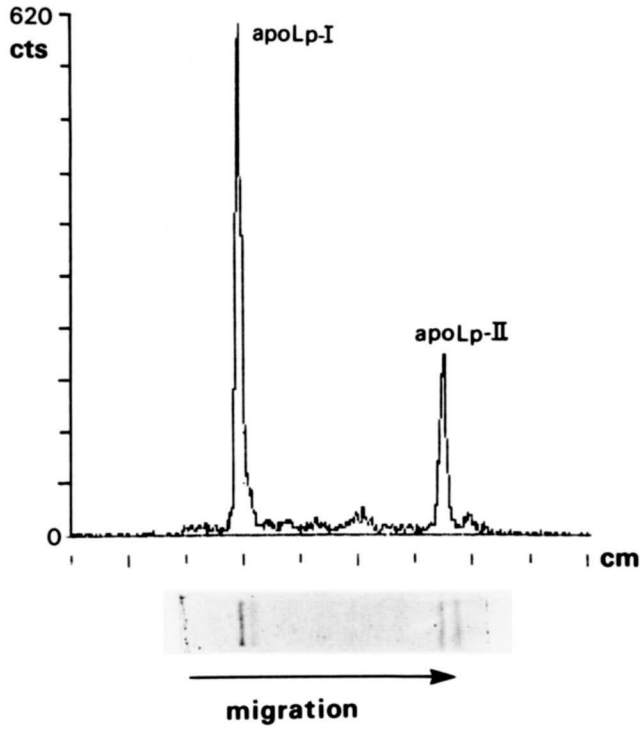

Fig. 3. Profile of radiolabel incorporation into larval or adult locust fat body proteins. Proteins precipitated from the fat body incubation medium with α-apoLp-I and α-apoLp-II serum were subjected to SDS-PAGE (7%), blotted onto nitrocellulose, and stained for proteins; the radioactivity incorporated into proteins was measured with a linear radioactivity analyzer.

shows that the radiolabel was incorporated almost exclusively into apoLp-I and apoLp-II. Similar results were obtained when the gels were exposed to fluorography (not shown). These results clearly demonstrate that the fat body synthesizes both apoLp-I and apoLp-II.

Density of secreted apoproteins

Since the fat body, in addition to being the site of apoprotein synthesis, is the center for synthesis and storage of lipids, it may be assumed that the newly synthesized apoproteins will be associated with lipids derived from the fat body and released into the hemolymph as lipoprotein particles. To test this hypothesis we determined the density of the protein fractions containing the released apoproteins after de novo synthesis by the fat body. In spite of the washing steps carried out after dissection, the fat body may contain adhering HDLp, tightly attached to binding sites (25). If released in fair amounts, this adhering HDLp may interfere in the density determination of the newly synthesized apoproteins. To evaluate the importance of this possible interference of HDLp, the kinetics of the release of adhering HDLp was studied after inhibition of the synthesis and subsequent release of newly synthesized apoproteins. Since both apoproteins comprise asparagine-linked oligosaccharides (26, 27), the possible inhibition by tunicamycin, which is known to inhibit N-glycosylation of proteins, was estimated. Fat bodies were incubated in the presence of tunicamycin throughout metabolic labeling, whereafter apoproteins were immunoprecipitated. The radioactivity in the precipitate was measured and compared with fat body control incubations without tunicamycin. The addition of tunicamycin resulted in a strong reduction of apoprotein synthesis or release since labeled apoproteins were virtually absent in the medium (**Table 1**). Based on this result it was possible to estimate the release of adhering HDLp. Fat bodies were incubated in vitro in saline containing tunicamycin for 4 h. Each hour the total amount of released HDLp protein was measured by ELISA and compared to fat body control incubations in the absence of tunicamycin. The results are shown in **Fig. 4**. In the first hour of the tunicamycin experiment the release of HDLp protein reached values of 7 μg/h whereas during continued incubation HDLp protein release decreased dramatically to ~30 ng/h. In contrast, the control fat bodies released ~9 μg/h in the first hour which decreased to a constant level of 2 μg/h. During the first hour, the released apoproteins therefore consisted mainly of adhering HDLp which is released almost completely during this period. During the following incubation periods, all apoproteins recovered seem to be derived from newly synthesized and released apoproteins.

Since a 1-h incubation results in the total release of adhering HDLp, adult and larval fat bodies were first preincubated in saline for 1 h prior to the 3-h incubation in fresh saline with radiolabeled amino acids. Medium obtained after this incubation was subjected to density gradient ultracentrifugation. The gradient was examined for protein quantity and distribution of radiolabel whereas the presence of apoLp-I and apoLp-II in all fractions was measured by ELISA. Profiles thus produced were compared with those obtained when hemolymph of adult locusts was similarly subjected to density gradient ultracentrifugation. As is

TABLE 1. Effect of tunicamycin on the amount of radiolabeled apoproteins recovered in the medium

Incubation Medium	Secreted Apoproteins
	dpm
+ Tunicamycin	3,150 ± 990
– Tunicamycin	47,910 ± 8,360

Adult fat bodies were incubated in vitro for 3 h in the presence of tritiated amino acids and, where indicated, 15 μg/ml tunicamycin. Medium was collected and subjected to immunoprecipitation. The precipitate was dissolved and radioactivity was measured. Values represent the mean of three separate fat body incubations ± SD.

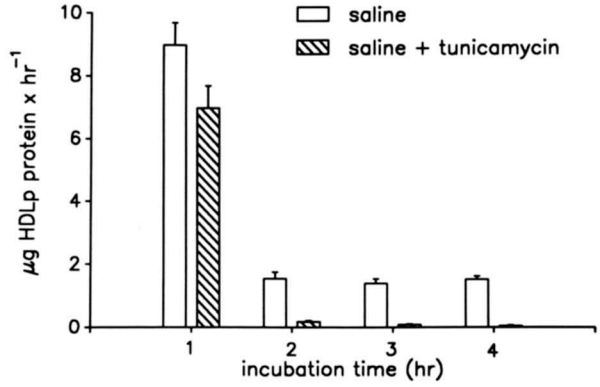

Fig. 4. Time-dependent release of HDLp proteins. Adult fat bodies were incubated for 4 h in saline in the presence or absence of tunicamycin; each hour the fat body was transferred to fresh saline. HDLp proteins released in medium were quantified by ELISA. Each histogram represents the mean of three separate fat body incubations (± SD).

shown in **Fig. 5A** for adult fat body, two major radioactivity peaks appeared at d 1.27 g/ml and 1.12 g/ml, respectively, implying synthesis of proteins in these fractions. Identical peaks were obtained for larval fat body incubations (not shown). The protein profile is identical with that obtained by subjecting hemolymph to density gradient ultracentrifugation; in the latter situation the peak at d 1.12 g/ml is HDLp (Fig. 5B). Therefore, the lipid-associated proteins at d 1.12 g/ml in Fig. 5A were also considered to be HDLp. Additional information from ELISA measurements provided evidence that the protein peak at d 1.12 g/ml is indeed HDLp. Using a mixture of MAbs specific for the apoproteins, apoLp-I and apoLp-II were clearly demonstrable in this fraction, both in medium and in hemolymph. No apoLp-I or apoLp-II could be detected in the major protein fraction at d 1.27 g/ml, indicating that newly synthesized apoproteins are not recovered as free apoproteins, but are first loaded with lipids and then released as HDLp.

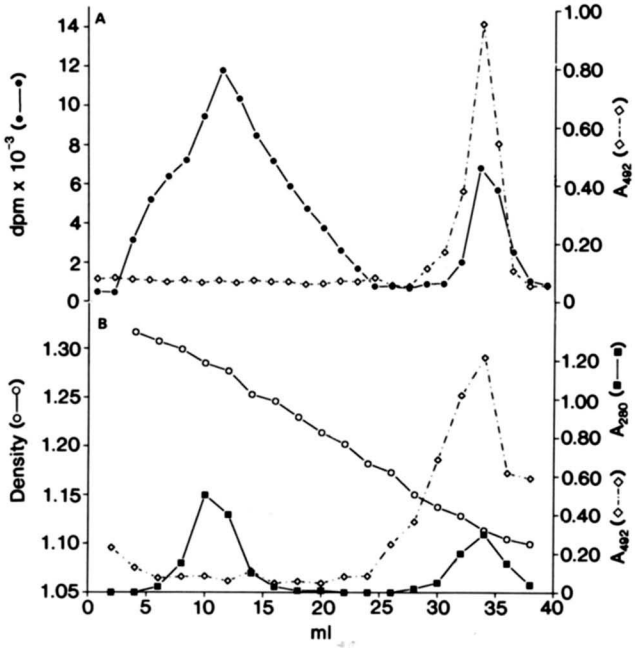

Fig. 5. Density gradient ultracentrifugation profile of incubation medium of fat bodies and of hemolymph. Adult fat bodies were incubated in the presence of tritiated amino acids and the collected media, as well as hemolymph of adult male insects, were subjected to density gradient ultracentrifugation. The final density gradient formed ranged from 1.09 to 1.31 g/ml. The gradient was fractionated into samples, which were examined for protein quantity (A_{280}), radioactivity, and assayed by ELISA for apoLp-I and apoLp-II (A_{492}). A: profile of collected media of adult fat body incubations. B: profile of hemolymph serving as a control.

Lipid analysis of newly synthesized HDLp

The lipid composition of HDLp, both synthesized in vitro by adult fat body and isolated from hemolymph, was determined by TLC and appeared to contain mainly diacylglycerol, sterol, hydrocarbons, and phospholipids (phosphatidylcholine and phosphatidylethanolamine, see **Fig. 6**). In addition, small amounts of

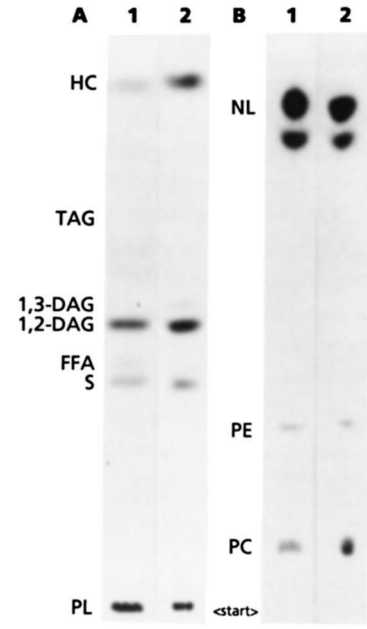

Fig. 6. Thin-layer chromatography of lipids from HDLp synthesized in vitro by adult fat body (after a 1-h preincubation for the removal of adhering HDLp) and HDLp isolated from hemolymph. HDLp was isolated by density gradient ultracentrifugation and the lipids were extracted with chloroform–methanol and subsequently separated by thin-layer chromatography. A: complete separation of lipid classes; lane 1, lipids extracted from HDLp synthesized in vitro; lane 2, lipids extracted from hemolymph HDLp. B: phospholipid separation; lane 1, lipids extracted from HDLp synthesized in vitro; lane 2, lipids extracted from hemolymph HDLp. HC, hydrocarbons; TAG, triacylglycerol; DAG, diacylglycerol; FFA, free fatty acid; S, sterol; PL, phospholipid; NL, neutral lipids; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

other lipid classes such as free fatty acids were present. Interestingly, HDLp synthesized in vitro contained only 1,2-DAG, as distinct from HDLp isolated from hemolymph in which both 1,2-DAG and 1,3-DAG were present. Since fat body releases DAG in the *sn*-1,2 configuration (28, 29) this is probably a result of a gradual conversion of 1,2-DAG into 1,3-DAG. The ratio between the lipid classes derived from hemolymph HDLp and HDLp synthesized in vitro was quantified by densitometry. In **Table 2** the relative amounts of lipids present in HDLp derived from both sources are shown, indicating that the distribution of lipid classes is very similar in both HDLps.

DISCUSSION

The data presented in this report clearly demonstrate that the fat body of the migratory locust, *Locusta migratoria*, synthesizes HDLp, the lipid-transporting protein in insects. Until now, only incomplete data were available (30, 31). We focused our study on apoLp-I and apoLp-II which constitute the protein moiety of HDLp.

The fat body of both larval and adult insects synthesized apoLp-I and apoLp-II with molecular masses of approximately 220 and 72 kDa, respectively. Both apoproteins were released into the incubation medium used in the in vitro system. However, the released apoproteins were not recovered in the medium as free apoproteins, but they appeared to be associated with lipids. By density gradient ultracentrifugation, using an ELISA for detecting apoLp-I and apoLp-II, we determined the density of the fractions containing the secreted apoproteins. The apoproteins were found only at d 1.12 g/ml, which is known to be the density of HDLp present in locust hemolymph (cf. 3). The lipids of the newly synthesized and secreted HDLp showed a composition that corresponds well with the lipid composition of the HDLp circulating in the hemolymph of resting locusts. The difference found in the hydrocarbon fraction may be due to the fact that the oenocytes, which probably are responsible for hydrocarbon synthesis, are located in peripheral fat body (32, 33), as in our in vitro fat system this may lead to a less efficient loading of hydrocarbons. However, the lower hydrocarbon content did not affect the density. Moreover, some heterogeneity in lipid composition between newly synthesized HDLp and circulating HDLp is possibly due to the lipid exchange between the latter HDLp and various tissues. The sterol demonstrated in in vitro secreted HDLp is apparently also descended from the fat body, although insects cannot synthesize the sterol ring and are dependent on dietary sterol (cf. 2). Apparently the fat body contains some storage sterols that are adequate for the assembly of HDLp.

TABLE 2. Lipid composition of HDLp synthesized and released by fat body in vitro or isolated from locust hemolymph

Lipid Class	Proportional Amount in HDLp from	
	In Vitro Release	Hemolymph
	%	
Hydrocarbons	18	27
Triacylglycerol	2	1
Diacylglycerol	36	38
Free fatty acid	4	2
Sterol	15	13
Total phospholipids	25	19
Phosphatidylcholine	(73)	(85)
Phosphatidylethanolamine	(27)	(15)

Lipids isolated from lipophorin synthesized and released by adult fat body in vitro were separated by TLC, visualized (see Fig. 6), and quantified by densitometry (n = 3). For comparison, HDLp isolated from hemolymph was also analyzed.

Our present data strongly support the view that apoLp-I and apoLp-II, after being synthesized, are loaded with lipid in the fat body to form a lipoprotein particle and are released into the medium as HDLp. Prasad et al. (13) proposed a different model for lipophorin biosynthesis, showing that in larval *Manduca sexta* a nascent particle with d 1.27 g/ml consisting of apoLp-I, apoLp-II, and phospholipid is synthesized and released by the fat body. This particle, referred to as very high density lipophorin, is then loaded with dietary lipids derived from the midgut to produce HDLp. In the locust, no evidence for such a mechanism exists, since neither free apoproteins nor very high density lipophorin are detectable and only HDLp is found in the medium. According to our model, no nascent lipophorin will be produced, as apoprotein synthesis and lipid loading are provided in the same tissue. Additional evidence for this model has been provided by Venkatesh et al. (14), who reported the secretion of lipophorin by the fat body in larvae of the southwestern corn borer, *Diatraea grandiosella*. Density and lipid composition of secreted lipophorin appeared to be similar to that of circulating lipophorin, suggesting secretion of mature HDLp. However, it remained possible that this mechanism was related to the period of non-feeding (diapause) of the larvae, which would prevent lipid uptake in the midgut, as suggested by Ryan (5). Recently, in vitro biosynthesis of lipophorin in larval *Musca domestica* was reported. The density of secreted lipophorin was identical with that of circulating lipophorin (34), which again is in accordance with the model proposed in the present paper for the locust.

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