Lipid transfer particle in locust hemolymph: purification and characterization

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Abstract A lipid transfer particle (LTP) from the hemolymph of adult male locusts, *Locusta migratoria*, was isolated and purified. The locust LTP exhibited its capacity to catalyze the exchange of diacylglycerol between low density lipophorin (LDLp) and high density lipophorin (HDLp). Contrary to the LTP reported for the tobacco hornworm, *M. sexta*, (4–6), the locust LTP appeared to lack the capacity to promote net transfer of diacylglycerol to form an intermediate density lipophorin, although it seems premature to conclude the complete lack of such a capacity in locust LTP. The original concentration of LTP in hemolymph is assumed to be extremely low compared to that of lipophorin; only a catalytic amount of LTP may be present in the hemolymph (e.g., only 160 μg of LTP was obtained from the original hemolymph containing 400 mg protein). The molecular weight of intact LTP was estimated to be about 600,000 and the LTP was comprised of three glycosylated apoproteins, apoLTP-I (mol wt 310K), apoLTP-II (mol wt 89K), and apoLTP-III (mol wt 68K). The locust LTP contained significant amounts of lipids; the total lipid content amounted to 14.4% and the lipids were comprised of 17% hydrocarbons, 44% diacylglycerol, 8% cholesterol, 13% free fatty acid, and 18% phospholipids. The above molecular properties of locust LTP are essentially similar to those reported for *M. sexta* LTP. —**Hirayama, Y., and H. Chino.** Lipid transfer particle in locust hemolymph: purification and characterization. *J. Lipid Res.* 1990. **31:** 793–799.

Supplementary key words lipophorin • exchange of diacylglycerol • net transfer of diacylglycerol • high density lipophorin • low density lipophorin

Lipophorin is the major lipoprotein of insect hemolymph and functions as a reusable shuttle to transport diacylglycerol, cholesterol, and hydrocarbons between tissues (1, 2). Concerning the functional feature of lipophorin, it has been proposed that loading and unloading of a certain lipid by lipophorin takes place at the cell surface of the target tissue; e.g., loading of diacylglycerol occurs at the fat body while unloading of this lipid takes place at the flight muscle, and the loading and unloading of hydrocarbons at the oenocyte and cuticle, respectively (2). Hence, lipophorins with different densities should coexist in insect hemolymph. However, lipophorin particles are usually found to be homogeneous in density as long as lipophorin is prepared from hemolymph of insects at the same developmental stage (2, 3), implying that all lipophorin particles are loaded with practically an equal amount of lipids. It is, therefore, possible that the lipids are, in vivo, rapidly transferred between lipophorin particles so that an equilibrium is attained, thereby resulting in homogeneity of density. In fact, Ryan et al. (4, 5) have presented several lines of evidence indicating the existence of a lipid transfer factor (particle) in the larval hemolymph of the tobacco hornworm, *Manduca sexta*, which has the capacity to catalyze the transfer of diacylglycerol between lipophorin particles having different densities. This factor stimulates the net transfer of diacylglycerol from a donor lipophorin (LDLp, obtained from adult hemolymph after injection of adipokinetic hormone) of density 1.03 g/ml to an acceptor lipophorin (HDLp, obtained from larval hemolymph) of density 1.18 g/ml, resulting in the formation of a new lipophorin fraction having an intermediate density. More recently, they have shown that the lipid transfer particle (LTP) of *M. sexta* is also capable of catalyzing the exchange of diacylglycerol between the two lipophorins, HDLp and LDLp (6, 7).

Since lipophorin is known to exist in the hemolymph of almost all insect species (1), it is important to test whether a factor equivalent to LTP of *M. sexta* exists in the hemolymph of other insect species. In this report we will present several lines of evidence indicating the existence of such a factor, namely lipid transfer particle (LTP), in the hemolymph of adult locusts. The purification and characterization of the locust LTP will also be described in comparison to the LTP of *M. sexta* reported by Ryan et al. (5, 6).

Abbreviations: LTP, lipid transfer particle; HDLp, high density lipophorin; LDLp, low density lipophorin; AKH, insect adipokinetic hormone; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Animal and hemolymph collection

Adult locust (3–6 weeks after final molt), *Locusta migratoria*, were taken from colonies maintained in this laboratory. Hemolymph was collected from locusts by flushing the hemocoele with saline (0.15 M NaCl, 5 mM EDTA, PIPES buffer, pH 6.8). The pooled hemolymph was centrifuged at 2,000 *g* for 5 min to remove the hemocytes.

Chemicals

Insect adipokinetic hormone (AKH) was obtained from Peninsula Laboratories (San Carlos, CA). [1-^{14}C]Palmitic acid (50 mCi/mmol) was purchased from Amersham, England. DEAE-Sepharose CL-6B was from Pharmacia. The following standard molecular weight marker proteins were obtained from Bio-Rad Laboratories (Richmond, CA); myosin (200,000), β-galactosidase (116,250), phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700). All other chemicals were of analytical grade. Double-glass-redistilled water was used throughout.

Preparation of lipophorins

High density lipophorin (HDLp) was prepared from the hemolymph of resting male locusts, according to a specific precipitation method of Chino and Kitazawa (8). Low density lipophorin (LDLp) was prepared from the hemolymph of AKH-injected male locusts (10 pmol AKH/insect) by KBr density gradient ultracentrifugation essentially according to Shapiro, Keim, and Law (9) as described by Chino, Downer, and Takahashi (10). In some experiments, KBr density gradient method was also used for the purification of HDLp. Both HDLp and LDLp preparations were dialyzed several times against saline (0.15 M NaCl, 1 mM EDTA, 10 mM PIPES buffer, pH 7.0) before use.

Labeling of diacylglycerol associated with HDLp and LDLp

To label HDLp, resting locusts were injected with [1-^{14}C]palmitic acid (1×10^6 dpm/insect) 90 min before hemolymph collection. For the labeling of LDLp, AKH (10 pmol/insect) was injected immediately after the injection of radioactive palmitic acid. Ninety minutes later, HDLp and LDLp were purified by the above-mentioned method. Both lipophorins will be called "labeled HDLp" and "labeled LDLp", respectively, in this report. In order to confirm that diacylglycerol is mainly or almost solely the labeled lipid in these two lipophorins, the lipids extracted from the labeled lipophorins were fractionated by Florisil column chromatography (11), and radioactivity in each fraction was determined. The data revealed that more than 95% of the radioactivity associated with labeled HDLp and LDLp was attributed to the diacylglycerol fraction.

Assay method for lipid transfer particle

One hundred μl of labeled HDLp (containing 0.2 mg protein and ca. 60 μg diacylglycerol) was incubated with 100 μl of unlabeled LDLp (containing 0.2 mg protein and ca. 360 μg diacylglycerol) at 30°C. The reaction was initiated by adding 50 μl of the LTP fraction. One hour later, the incubation mixture was placed in ice, and saline (0.15 M NaCl, 1 mM EDTA, 10 mM PIPES buffer, pH 7.0) was added to bring the final volume to 5 ml; the mixture was then subjected to KBr density gradient ultracentrifugation. After ultracentrifugation, the yellow lipophorin band corresponding to LDLp was collected by means of a Pasteur pipet and assayed for radioactivity transferred from labeled HDLp during the incubation.

Purification of lipid transfer particle (LTP)

In most experiments, the hemolymph (about 50 ml containing 350–400 mg protein) collected by the flushing method from 100 male locusts was used as the starting material. All procedures were done at 2°C.

Specific precipitation. Considering the fact that no exchange of diacylglycerol occurs if both donor and acceptor lipophorins are purified prior to the incubation (refer to Fig. 4A and Fig. 5A), it is suggested that LTP can be separated from lipophorin at a certain step during the purification procedure. Therefore, the specific precipitation method originally developed for the purification of lipophorin (8) was used as the initial step of the purification procedure.

The freshly collected hemolymph (about 50 ml) was dialyzed against large volumes of distilled water until the hemolymph solution became slightly turbid. After removing the turbidity by centrifugation at 10,000 *g* for 5 min, 8 volumes of ice-cold distilled water was added all at once to the supernatant in order to precipitate lipophorin and a minute amount of other proteins (8). After collecting the precipitate by centrifugation at 10,000 *g* for 5 min, both the precipitate and the supernatant were assayed for LTP activity as described above. The results indicated that the precipitate had significant activity while no activity was detected in the supernatant even in the fraction concentrated by adding ammonium sulfate to 80% saturation.

Since the precipitate had LTP activity and since this fraction is known to consist mainly of lipophorin and a small amount of some other proteins (see Fig. 3 lane B), it is conceivable that LTP is coprecipitated with lipophorin and can be separated from lipophorin by the subsequent step of DEAE-cellulose column chromatography which has been used as the final step in the purification of lipophorin (8). We then tried to use this ion exchange column chromatography as the second step for the purification of LTP.

DEAE-Sepharose column chromatography. The above precipitate was first dissolved in a small volume of saline (0.2 M NaCl, 2 mM EDTA, 2 mM PMSF, 20 mM PIPES buffer, pH 7.0). The solution was diluted by adding an equal volume of distilled water and applied to DEAE-Sepharose column (1.0 × 15 cm) equilibrated with a diluted saline (0.1 M NaCl, 1 mM EDTA, 1 mMPMSF, 10 mM PIPES buffer). The column was first run with 100 ml diluted saline to elute lipophorin (HDLp) and subsequently run with a linear concentration gradient of 0.1–0.4 M NaCl (in diluted saline). The elution profiles of the protein and LTP activity are illustrated in **Fig. 1** and demonstrate that HDLp and LTP are completely separated from each other by this chromatographic procedure, and that LTP activity was found in the fractions eluted with 0.15–0.25 M NaCl. The active fractions (about 25 ml in total) were combined and subjected to a density gradient ultracentrifugation for further purification.

Density gradient ultracentrifugation. The active fraction (about 25 ml) was first adjusted to 30 ml by adding diluted saline, to which solid KBr was then added to give a final density of 1.31 g/ml. The solution thus prepared was placed into two tubes (about 16.5 ml in each tube) and overlayered with an equal volume of KBr-saline adjusted to 1.10 g/ml. The tubes were then centrifuged at 50,000 rpm for 16 h at 4°C in a Hitachi ultracentrifuge (model 70p-72). After centrifugation, the sample was fractionated by a density gradient fractionator (ISCO, model 640). Each sample (1 ml) was monitored for protein at 280 nm and an aliquot (50 μl) was assayed for LTP activity. The results (**Fig. 2**) demonstrate that the maximum LTP activity appears at the density of 1.22 g/ml and coincides exactly with the absorbance peak at 280 nm. The active

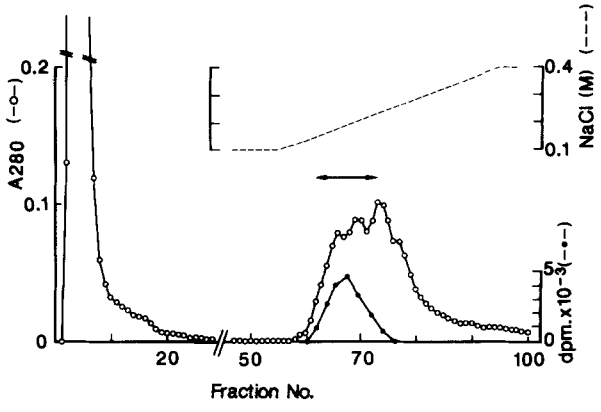

Fig. 1. Elution profile (DEAE-Sepharose column chromatography) of the fraction precipitated at step 1. The column was run at the flow rate of 7 ml/h and 2 ml was collected in each tube. An aliquot (50 μl) of each fraction was assayed for LTP activity as described in Methods. The LTP activity is expressed as the radioactivity found in LDLp fraction after density gradient ultracentrifugation (see Methods). The large absorbance peak at 280 nm appearing before the NaCl concentration gradient elution represents the lipophorin fraction (HDLp).

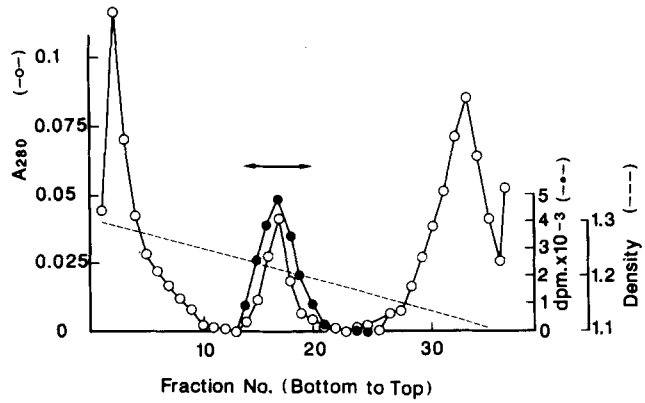

Fig. 2. Profile on density gradient ultracentrifugation (d 1.10–1.31 g/ml) of LTP fraction obtained by DEAE-Sepharose column chromatography. Tubes were fractionated (1-ml fractions) from the top. Each fraction was monitored at 280 nm and an aliquot (50 μl) was assayed for LTP activity. Other explanations as in Fig. 1.

fractions were combined, dialyzed against diluted saline, and subjected to native PAGE to test its homogeneity. As shown in **Fig. 3**, lane C, the preparation gave a single band on the gel, revealing its high homogeneity. Starting with the hemolymph of 100 male locusts (about 400 mg protein), 160 μg protein of LTP was obtained at this final step of purification. The purified LTP could be stored at 0°C without significant loss of its activity for at least 1 week.

Lipid determination

The lipids were extracted from purified LTP with chloroform–methanol 2:1 (v/v) and analyzed by an Iatroscanner (model Th-10) essentially according to the method of Ackman (12) as described by Chino, Kiyomoto, and Takahashi (13). Diacylglycerol content on HDLp and LDLp was also determined by the same procedure.

Gel electrophoresis

Native and SDS-polyacrylamide gel electrophoreses of purified LTP were performed according to the method of Davis (14) and the method Laemmli (15), respectively. The gels were stained with Coomassie blue.

Determination of protein and radioactivity

Protein was determined by the method of Lowry et al. (16). Radioactivity was counted by a liquid scintillation counter (LKB, model 1219) after dissolving the samples in Aquasol-2.

RESULTS

As we already knew from the earlier report of Ryan et al. (4, 5) that a factor in the *M. sexta* hemolymph catalyzes a net transfer of diacylglycerol between LDLp and HDLp

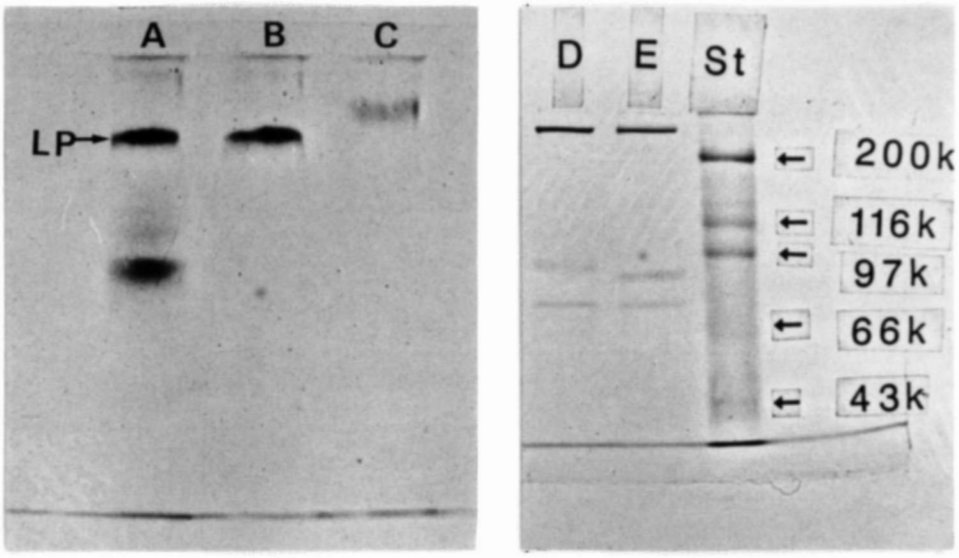

Fig. 3. Native and SDS-polyacrylamide gel elecrophoreses of locust LTP. Left, native PAGE (4% gel) with 10 μg protein in each lane; right, SDS-PAGE (7% gel) with 5 μg protein in each lane. Lane A, whole hemolymph from resting male locust; lane B, fraction precipitated under low ionic concentration at step 1 (main band represents HDLp); lane C, purified LTP; lane D, purified LTP treated with SDS in the presence of mercaptoethanol; lane E, purified LTP treated with SDS in the absence of mercaptoethanol; St, standard marker proteins (myosin, 200K; β-galactosidase, 116K; phosphorylase b, 97K; BSA, 66K; ovalbumin, 43K); Lp, lipophorin (HDLp).

to form a new lipophorin having an intermediate density, we initially tried to reproduce this phenomenon using *Locusta migratoria*. After many unsuccessful attempts, we came across a factor that catalyzes, not net transfer, but the exchange of diacylglycerol between LDLp and HDLp.

Evidence indicating the existence of a factor in hemolymph that catalyzes the exchange of diacylglycerol between HDLp and LDLp

Definite evidence indicating the existence of such a factor (LTP) in the hemolymph of adult locusts is given in **Fig. 4** and **Fig. 5**. When labeled LDLp was incubated with unlabeled HDLp in the presence of purified LTP for 3 h, it was found that significant radioactivity was transferred from labeled LDLp to unlabeled HDLp during incubation (Fig. 4B). The reverse transfer of radioactive diacylglycerol was also demonstrated by the incubation of labeled HDLp with unlabeled LDLp in the presence of LTP (Fig. 5B). Contrary to the above results, when labeled donor and unlabeled acceptor lipophorins were incubated in the absence of LTP, such a transfer was never observed in either direction between LDLp and HDLp, indicating that no spontaneous transfer of diacylglycerol occurs during the 3-h incubation (Figs. 4A and 5A). The results illustrated in Figs. 4 and 5 also demonstrate that no new lipophorin fraction having an intermediate density is formed during incubation. This implies that no net transfer of diacylglycerol takes place in either direction between LDLp and HDLp. In fact, no significant changes in the diacylglycerol content of LDLp (180 ± 12 μg di-

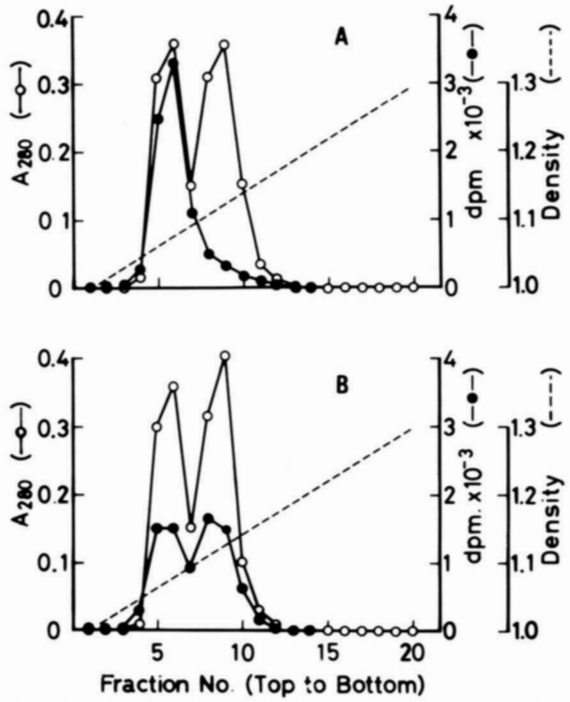

Fig. 4. Transfer of labeled diacylglycerol from LDLp to HDLp. One hundred μl of labeled LDLp containing 0.2 mg protein and about 9,000 dpm diacylglycerol was incubated at 30°C for 3 h with 100 μl of unlabeled HDLp containing 0.2 mg protein in the presence of 50 μl of purified LTP containing 0.5 μg protein. After incubation, the mixture was brought to 5 ml by adding saline and subjected to KBr density gradient ultracentrifugation. After centrifugation for 4 h, the tubes were fractionated in 0.5-ml fractions from the top. The lipophorin fractions were monitored at 280 nm, and the radioactivity in each fraction was determined as described in Methods. A, incubation in the absence of LTP (control); B, incubation in the presence of LTP.

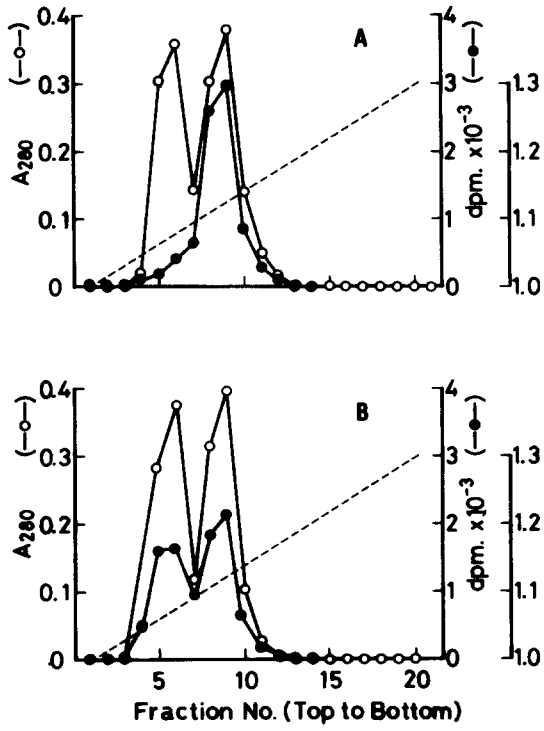

Fig. 5. Transfer of labeled diacylglycerol from HDLp to LDLp. One hundred μl of labeled HDLp containing 0.2 mg protein and about 8,500 dpm diacylglycerol was incubated with 100 μl of unlabeled LDLp containing 0.2 mg protein in the presence of 50 μl of LTP containing 0.5 μg protein. Other explanations as in Fig. 4. A, incubation in the absence of LTP; B, incubation in the presence of LTP.

acylglycerol/100 μg protein, three determinations) and HDLp (30 μg ± 2.2 diacylglycerol/100 μg protein) were observed before or after incubation.

Calculating from the specific radioactivity of donor HDLp (170 dpm/μg diacylglycerol) and the radioactivity (5,540 dpm) found in acceptor LDLp after incubation, the purified LTP mediates an exchange of approximately 55 μg diacylglycerol/μg LTP per h in a standard assay system using 0.6 μg LTP.

Partial characterization of locust LTP

The lipid composition of purified LTP was determined by Iatroscanner. The results are given in **Table 1**. The data for the lipid components of lipophorin (HDLp) are also given for comparison. The total lipid content of LTP amounted to about 14% of its total weight. This value is consistent with the observed density (1.22 g/ml). Diacylglycerol represents the major acylglycerol in LTP as well as in lipophorin. The content (percentage of total lipid) of hydrocarbons and free cholesterol is nearly equal to that of lipophorin. However, a big difference exists in the content of free fatty acid and phospholipids; a significant amount of free fatty acid is detected in LTP but not in lipophorin, and phospholipid content in LTP is considerably lower than that in lipophorin.

The molecular weight of native LTP was estimated by gel permeation chromatography on Sepharose CL-4B column (1 × 60 cm), which was run with saline (0.15 M NaCl, 1 mM EDTA, 1 mM PMSF, 10 mM PIPES buffer, pH 7.0). The LTP fraction was localized by the assay of LTP activity. For reference, purified HDLp was also applied to a column of the same size that ran under the same conditions. Although the data are not shown, the elution profile indicated that the peaks of LTP fraction and of HDLp were inseparable; the molecular weight of LTP is nearly equal to that reported for HDLp (8), approximately 600K.

The subunit structure of pure LTP was examined by SDS-PAGE (Fig. 3). The locust LTP is composed of three different apoprotein components, apoLTP-I (mol wt, 310K), apoLTP-II (mol wt, 89K), and apoLTP-III (mol wt 68K). All three apoproteins stained positively by the PAS-staining method (16), suggesting that the apoproteins are glycoproteins. The gel pattern was identical regardless of the addition of a sulfhydryl reducing agent (compare lane D to E in Fig. 3). This indicates the lack of disulfide bonds in LTP. Although there appears to be a slight difference in mobility of apoLTP-II between the samples treated with and without mercaptoethanol, we have not investigated this problem further. The data of the three apoproteins, taken together with the molecular weight (about 600K) of intact LTP and the value (14%) of the total lipid content of LTP (Table 1), imply that intact LTP consists of one apoLTP-I, one apoLTP-II, and one apoLTP-III molecule. The *M. sexta* LTP has been assumed to contain two apoLTP-I, two apoLTP-II, and one or two apoLTP-III molecules (6). This discrepancy, however, may depend on the difference in the estimated molecular weights of the two intact LTPs, about 600K for locust LTP and more than 670K for *M. sexta* LTP. A more exact determination of the molecular weight of native LTP is necessary to resolve this problem.

TABLE 1. A typical analysis of lipid composition of locust LTP; the data of locust lipophorin (HDLp) are also shown for comparison

Components	% Weight	
	LTP	Lipophorin[a]
Protein	85.7	59
Total lipid	14.3	41
Hydrocarbons	2.4 (17.1)[b]	8.7 (21.2)
Triacylglycerol	ND[c]	0.7 (1.7)
Diacylglycerol	6.4 (44.4)	13.5 (33.0)
Cholesterol	1.2 (8.2)	3.3 (8.0)
Cholesteryl ester	ND	Trace
Free fatty acid	1.8 (12.6)	ND
Phospholipids	2.5 (17.7)	14.8 (36.1)

[a]From data of locust lipophorin (8).
[b]Values in parentheses are the percentages of total lipid.
[c]Not detectable.

DISCUSSION

Ever since the existence of a lipid transfer protein (LTP) in rabbit and human plasma was demonstrated by Zilversmit, Hughes, and Balmer (17) and Patnaik et al. (18), the research field of mammalian LTP has been making rapid progress. Mammalian LTP has a capacity to catalyze the net transfer as well as the exchange of lipids such as triacylglycerol and/or cholesteryl ester between HDL and LDL or VLDL (19). The first reported insect LTP, *M. sexta* LTP, appears to be functionally similar to mammalian LTP, although the molecular properties of the *M. sexta* LTP differ considerably from those of mammalian LTP; e.g., the estimated molecular weight of intact *M. sexta* LTP is at least 670K (6) and much larger than that (58K–66K) reported for mammalian LTP (20).

The data presented in this report indicate that locust LTP is essentially similar to *M. sexta* LTP in the following properties: apoprotein composition, density, and lipid composition, although the estimated molecular weight (600K) of intact locust LTP is lower than that (670K) of *M. sexta* LTP. The locust LTP as well as the *M. sexta* LTP (6) are composed of three glycosylated apoproteins, although there appear to be some differences in the molecular weight of apoLTP-III (68K for locust apoLTP-III, 55K for *M. sexta* apoLTP-III) and in the proposed molar ratio of each apoprotein (see Results). The lipid composition of locust LTP is also similar to that of *M. sexta* LTP. The total lipid content of the former amounts to about 14% which is very close to the value (13.8%) reported for the latter (6), although some differences do exist in lipid content between the two LTPs; e.g., phospholipid is the major lipid (60%) in *M. sexta* LTP while in locust LTP there is only 18% phospholipid, and triacylglycerol (5.6%) is found in *M. sexta* LTP but this lipid was not detected in locust LTP (no data are available for hydrocarbons in *M. sexta* LTP).

Although, as mentioned above, locust LTP appears to basically resemble the *M. sexta* LTP, the present study suggests that there may be a significant difference in the function. Ryan et al. (4–6) have demonstrated that under certain conditions, *M. sexta* LTP catalyzes the net transfer of diacylglycerol from LDLp to HDLp resulting in the formation of an intermediate density lipophorin, in addition to the exchange of this lipid between the two lipophorins. They have observed that the net transfer of diacylglycerol, via facilitated exchange, occurs when a mixture of 1 mg LDLp (containing 1,250 μg diacylglycerol) and 1 mg HDLp (containing 250 μg diacylglycerol) is incubated in the presence of higher concentration of LTP (higher than 0.3 μg) at a longer incubation time. In the case of locust, however, no intermediate density lipophorin was observed even when 0.2 mg LDLp (containing 360 μg diacylglycerol) and 0.2 mg HDLp (containing 60 μg diacylglycerol) were incubated in the presence of a large amount of LTP (more than 3 μg) for more than 3 h, although radioactivity reached an equilibrium between the donor and the acceptor lipophorins. This suggests that locust LTP, unlike *M. sexta* LTP, has no capacity to promote the net transfer of diacylglycerol between LDLp and HDLp. At present, however, it seems premature to conclude that locust LTP completely lacks such a capacity and, therefore, differs functionally from *M. sexta* LTP. Indeed, we have observed that when locust LTP is added to locust HDLp and the mixture is incubated for a long time (e.g., 3 h) and subjected to KBr density gradient ultracentrifugation, the peak of the lipophorin fraction becomes sharper compared to the peak before incubation or in the absence of LTP. This suggests that the locust LTP has a capacity to promote the net transfer of diacylglycerol between lipophorin particles so that the densities of the lipophorin particles become more homogeneous. More detailed studies, including those of the kinetic properties of locust LTP, are necessary to resolve this problem.

The yield of locust LTP at the final step of purification was only 160 μg although the starting material contained about 400 mg protein. However, the possibility that the locust LTP was not entirely precipitated under low ionic concentration at step 1 of the purification is ruled out as no LTP activity was detected in the supernatant. Since the total activity of LTP in the original hemolymph was not determined in this experiment, it is impossible to calculate the recovery of LTP throughout the purification steps. In the purification of *M. sexta* LTP, the recovery is calculated to be 54% and the yield of the final preparation is 800 μg starting with lipophorin-free hemolymph containing 1,650 mg protein (R. O. Ryan, personal communication). Considering that only a catalytic amount of LTP is needed for its function, it seems only natural that the original concentration of LTP in insect hemolymph is extremely low compared to that of lipophorin.

In connection with the function of insect LTP, it is particularly important to investigate whether LTP is also capable of transferring other lipids such as hydrocarbons and/or free cholesterol between lipophorin particles. The present study demonstrated that locust LTP contains such lipids in addition to diacylglycerol. This observation has stimulated our interest in this problem, which is now being investigated in this laboratory.

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