Diacylglycerol-carrying lipoprotein of hemolymph of the locust and some insects

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Abstract The diacylglycerol-carrying lipoprotein (DGLP) was purified from hemolymph of the locust, *Locusta migratoria*, by a rapid method which included a specific precipitation at low ionic concentration and DEAE-cellulose column chromatography. The final preparation was highly homogeneous as judged by gel electrophoresis, electron microscopy, and immunodiffusion. The locust DGLP molecule was almost spherical in shape with a diameter of about 130 Å. The molecular weight, determined by a sedimentation equilibrium method, was approximately 580,000. The total lipid content amounted to about 40%. The lipids comprised diacylglycerol (33% of total lipid), hydrocarbon (21%), cholesterol (8%), and phospholipids (36%). The hydrocarbon fraction contained a number of *n*-alkanes and methylalkanes ranging from C_{25} to C_{38} in chain length. Mannose (3%) and glucosamine (0.5%) were associated with the apoprotein of DGLP. Apoprotein of locust DGLP consisted of two subunits, heavy chain (mol wt 250,000) and light chain (mol wt 85,000); carbohydrate (mannose) was associated only with the heavy chain. Tests of physiological function of DGLPs from locust, cockroach, and silkworm suggest that the insect DGLP serves multiple roles as a true carrier molecule in transporting diacylglycerol, cholesterol, and hydrocarbon from sites of storage, absorption, and synthesis to sites where these lipids are utilized as metabolic fuel, precursors for triacylglycerol and phospholipid synthesis, or structural components of cell membrane and cuticle. In addition, the insect DGLPs displayed no species-specificity in terms of the functions, whereas they were immunologically distinguishable.—**Chino, H., and K. Kitazawa.** Diacylglycerol-carrying lipoprotein of hemolymph of the locust and some insects. *J. Lipid Res.* 1981. **22:** 1042–1052.

Supplementary key words lipid transport · lipoprotein · cholesterol · hydrocarbon

In insects, the predominant form in which fatty acids are transported from fat body is as diacylglycerol associated with a specific hemolymph lipoprotein (1). The initial reports by Chino and Gilbert (2, 3) for the *Cecropia* silkworm, the *Melanoplus* grasshopper, and the American cockroach have been confirmed by many investigators and extended to other species including locust (4). The specific hemolymph lipoprotein was first isolated from the pupal hemolymph of the *Philosamia* silkworm in our laboratory, and named diacylglycerol-carrying lipoprotein (DGLP) (5). We have demonstrated in vitro the capacity of purified DGLP to take up diacylglycerol specifically from the fat body, and have characterized extensively the molecular nature of this lipoprotein (5).

Recently, we developed a simple but efficient method to purify DGLP from hemolymph of the American cockroach, which is based on the method that was originally developed for the purification of DGLP from the *Philosamia* silkworm (6). We have now applied this new technique to the purification of DGLP from hemolymph of adult locusts with a slight modification, and have demonstrated its extreme efficiency in yielding a highly pure preparation of DGLP with satisfactory recovery. The method described in this report offers advantages over methods that have been employed by other investigators (7–9).

Our previous study (6) demonstrated in vitro that DGLP of the American cockroach has a capacity to take up diacylglycerol not only from the storage site of the fat body but also from the absorption site of the midgut (intestine). This observation has now been extended to DGLPs of the locust and the silkworm, and this report indicates that the DGLP molecule serves multiple functions as a true carrier transporting various lipids such as diacylglycerol, cholesterol, hydrocarbon, and carotenoid from their sites of storage, absorption, and synthesis to the sites where these lipids are utilized as metabolic fuel, precursors of triacylglycerol and phospholipid synthesis, or structural components of cell membrane and cuticle.

Therefore, in view of the functional multiplicity as a true carrier, the insect DGLP[1] is a unique lipoprotein and essentially different from mammalian plasma lipoproteins.

Abbreviations: DGLP, diacylglycerol-carrying lipoprotein; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; SDS, sodium dodecyl sulfate.

[1] We have recently proposed a new term "lipophorin" (in Greek: *lipos*, lipid; *phoros*, bearing) as an appropriate generic term for this important class of insect hemolymph lipoprotein (27).

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MATERIALS AND METHODS

Animals and collection of hemolymph

Adult locusts, *Locusta migratoria* (2–5 weeks after final molt) and adult American cockroaches, *Periplaneta americana*, were taken from colonies maintained in this laboratory. The diapausing (dormant) pupae of the silkworm, *Philosamia cynthia*, were collected from the field. The method of feeding insects on radioactive compounds is given in an earlier report (10). Hemolymph was collected from locusts and cockroaches by a "washing out method" that is described in detail in a previous paper (6).

Chemicals

Chromatographically pure phosphatidylcholine was prepared from egg yolk using silicic acid column chromatography. [1-^{14}C]Palmitic acid (50 mCi/mmol) and [4-^{14}C]cholesterol (53 mCi/mmol) were purchased from New England Nuclear Corp. Florisil (60–100 mesh) and DEAE-cellulose were obtained from Sigma Chemical Co. All other chemicals including standard sugars were of analytical grade and solvents were redistilled as appropriate. Glass-redistilled water was used throughout the experiments.

Purification of DGLP from locust hemolymph

The method of purification of DGLP from locust hemolymph was basically similar to that developed for purifying DGLP from the American cockroach (6). As emphasized in the previous report (6), the collection of hemolymph by a "washing out" method as well as the subsequent biochemical procedures are essential for the purification of DGLP. The freshly collected hemolymph (hemocyte-free) from male or female adult locusts (usually 5–10 animals for each run) was dialyzed against cold distilled water for about 40 min (male) or 15 min (female). The dialysis was stopped when the solution in the dialysis-tube became slightly turbid. After the turbidity was removed by centrifugation at 10,000 *g* for 5 min, eight volumes of ice cold distilled water was added *all at once* to the supernatant (pH adjustment was unnecessary at this step, but slow addition of cold distilled water caused an irreversible aggregation of DGLP). The solution was left for 10 min in an ice bath to allow precipitation of DGLP and related protein fractions. The suspension was centrifuged and the precipitate was redissolved in 0.5–1.0 ml of 0.2 M phosphate buffer, pH 6.0. The solution was diluted by adding an equal volume of distilled water and applied to a column (6 × 1.1 cm) of DEAE-cellulose equilibrated with 0.1 M phosphate buffer, pH 6.0. The DGLP fraction passed through the column with the same buffer and a second protein fraction was eluted subsequently with 0.25 M KCl in 0.05 M phosphate buffer, pH 6.0 (see Fig. 1).

Lipid extraction, separation, and determination

Neutral lipid classes and free fatty acids were extracted from hemolymph, lipoprotein fractions, or incubation media by the procedure of Dole (11) and were separated on columns of Florisil (12). The total lipids including phospholipids were extracted from DGLP with chloroform–methanol 2:1 (v/v). The amounts of hydrocarbon, cholesteryl ester, cholesterol, tri-, di-, and monoacylglycerols, and phospholipid were determined by gravimetry, colorimetry, or scanning, according to the procedures described in earlier communications (5, 13). The hydrocarbon fraction obtained after Florisil column chromatography was submitted to further analyses with GLC and GLC–MS using Shimadzu, GC-4CM and Hitachi, RMU-6M. The conditions of GLC–MS have been given in the previous paper (6).

Sugar, amino sugar, and amino acid

The sugar and amino sugar present in DGLP were analyzed by GLC after delipidation of DGLP with chloroform–methanol 2:1 (v/v). Detailed conditions are given in an earlier paper (13). The amount of amino sugar was determined by the method of Blix (14) following hydrolysis of the delipidated DGLP in 4 N HCl at 100°C for 6 hr. Amino acid composition of the delipidated DGLP was determined on an automatic amino acid analyzer (JEOL, model JLC-6AH) after hydrolysis with 6 N HCl at 100°C for 24 hr.

Gel electrophoresis

Polyacrylamide gel electrophoresis of the purified DGLP was performed in 3.75% gel. The gel was stained with Coomassie blue G-250 by a rapid staining method (15). To analyze the subunit structure of the apoprotein of DGLP, the delipidated DGLP was run on SDS 5% polyacrylamide gel electrophoresis after the method of Weber and Osborn (16). After electrophoresis, the gels were stained with Coomassie blue for protein and were stained for carbohydrate by the periodic acid-Schiff (PAS) method (17).

Molecular weight determination

The molecular weight of the purified DGLP (dissolved in 0.1 M KCl with 0.05 M phosphate buffer, pH 6.0) was determined by the rapid sedimentation equilibrium method of Yphantis (18), in a Beckman model E analytical ultracentrifuge equipped with a photoelectric scanning system. The equilibrium was established at 13,500 rpm after a 23-hr run at 15°C.

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Preparation of antibody and immunodiffusion test

The purified locust DGLP emulsified with Freund's complete adjuvant was injected subcutaneously into several sites on the back of a rabbit. A booster injection was given 2 weeks after the initial injection. Twelve days later, the rabbit was bled and IgG was separated from serum by ammonium sulfate precipitation at 40% saturation. After dialysis against Tris-buffered saline (0.02 M Tris-HCl containing 0.15 M NaCl, pH 7.5), the anti-DGLP IgG thus obtained was stored frozen at −20°C. Immunodiffusion tests were carried out after the method of Ouchterlony (19) using 1% agarose gels buffered with the above Tris-buffered saline. After antigens and antibody were allowed to diffuse for 24 hr at 15°C, the gel plates were examined for precipitin lines.

Uptake of diacylglycerol in vitro

The capacity of the DGLP to accept diacylglycerol from the fat body and midgut was demonstrated by incubating prelabeled fat body or midgut in a Ringer solution containing purified DGLP. After incubation, the radioactivity of diacylglycerol released into incubation media was determined. The details of preparing prelabeled fat body and of determining diacylglycerol uptake have been described in an earlier communication (5). The preparation of prelabeled midgut and the assay method are also given in a previous paper (6).

Radioassay

Aliquots of protein fractions for radioassay were transferred to vials containing 10 ml of Insta-gel (Packard). When radioassayed for lipid fractions, the samples were dissolved in 8 ml of cocktail (1 liter toluene containing 3 g of PPO and 100 mg of dimethyl POPOP). Radioactivity was counted on a Packard liquid scintillation counter, model 3330.

RESULTS

Purification of locust DGLP

Fig. 1 illustrates the elution from a DEAE-cellulose column of fractions derived from the pooled hemolymph of female locusts previously injected with [1-^{14}C]palmitic acid. The major peak of radioactivity coincides with the major protein peak (fraction A) which is designated as DGLP. By contrast, the label in the second fraction (fraction B) is low. It is evident from our recent report (20) that the second fraction is vitellogenin (female-specific protein) that precipitates together with DGLP under low ionic concentra-

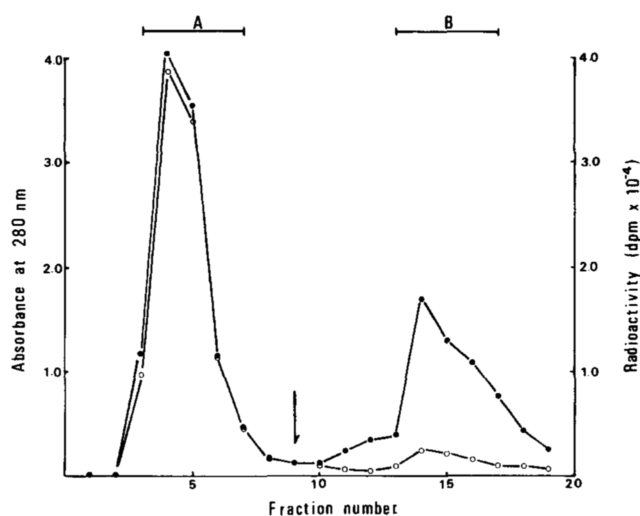

Fig. 1. DEAE-cellulose column chromatography of the fraction precipitated after adding 8 volumes of cold distilled water. Original labeled hemolymph was collected and pooled from six female locusts 4 hr after injection of [1-^{14}C]palmitic acid (about 1×10^6 cpm/animal). One-ml fraction was collected in each tube. Arrow, beginning of elution with 0.25 M KCl in 0.05 M phosphate buffer, pH 6.0; open circle, radioactivity; solid circle, absorbance at 280 nm.

tion but is completely separated from DGLP by DEAE-cellulose column chromatography (see also Fig. 2,a).

Elution profiles similar to those illustrated in Fig. 1 were obtained from the pooled hemolymph of male locusts, although much less protein was recovered as fraction B, because of the absence of vitellogenin in the male hemolymph (data not shown).

The purified locust DGLP was deep yellow in color due to the presence of carotenoid pigments as reported for DGLP-I of the *Philosamia* silkworm (5).

The homogeneity of the DGLP preparation was demonstrated by polyacrylamide gel electrophoresis of fraction A. Typical electropherograms shown in **Fig. 2** indicate that a single protein is eluted from DEAE-cellulose column following purification from male or female hemolymph. The homogeneity of the locust DGLP preparation was further demonstrated by immunodiffusion (**Fig. 3**). A single precipitin line was consistently formed when the anti-locust DGLP IgG was reacted with DGLP purified from male and female locusts, or with male and female hemolymph, confirming the high homogeneity of the DGLP preparation. The antibody cross-reacted neither with DGLP from the American cockroach nor with DGLP from the *Philosamia* silkworm (Fig. 3).

It was demonstrated (see Table 2) that the radiolabel of DGLP was predominantly associated with diacylglycerol, and, therefore, the efficiency of the purification procedure was tested by determining the radioactivity of the diacylglycerol fraction recovered

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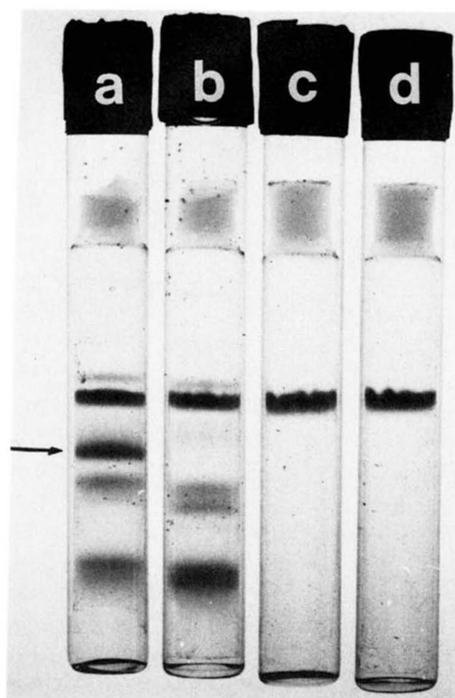

Fig. 2. Polyacrylamide gel electrophoreses: a, female locust hemolymph (arrow indicates vitellogenin); b, male locust hemolymph; c, DGLP purified from female locust; d, DGLP purified from male locust.

at each step of the purification from the pooled hemolymph of locusts that had been injected with [1-^{14}C]palmitic acid. The results are summarized in **Table 1** and demonstrate that the purification procedure yields about 70% or more recovery of DGLP from either male or female hemolymph. This recovery is considerably higher than that (33%) observed for the purification of DGLP from the American cockroach (6).

The data given in Table 1 permit calculation of the original content of DGLP in hemolymph, based on the protein amount; the percentage of DGLP in original hemolymph is about 35% (11 × 100/70 × 100/44.5) for males and 28% (9.7 × 100/74 × 100/47.1) for females. The reduced percentage of DGLP in female hemolymph is probably due to the presence of vitellogenin (see Fig. 2, a).

Table 2 indicates that more than 90% of the radioactivity is associated with the diacylglycerol fraction when the DGLP is prelabeled by injection with [1-^{14}C]palmitic acid prior to the collection of hemolymph. The label associated with other lipid fractions, except for hydrocarbon, is negligible.

Electron microscopy and molecular weight of locust DGLP

A negatively stained electron micrograph of the purified DGLP of locust is illustrated in **Fig. 4** and it demonstrates high homogeneity in terms of molecular shape and size; the molecule is almost globular with a diameter of 131 Å ± 11.

The molecular weight of locust DGLP was determined by a sedimentation equilibrium method. Since the total lipid content and the amino acid composition of locust DGLP (see the next section) resembled those reported for DGLP (DGLP-I) from the *Philosamia* silkworm, 0.87 was taken as the specific partial volume which had been theoretically calculated for the silkworm DGLP (5). Thus, the molecular weight of locust DGLP was estimated to be approximately 580,000, which is nearly equal to the molecular weight (600,000) reported for cockroach DGLP (6).

Chemical composition of locust DGLP

The typical data of the lipid composition of locust DGLP, together with the data from cockroach DGLP (6) and silkworm DGLP (5) for comparison, are given in **Table 3.** The total lipid content of locust DGLP amounts to about 40%. Diacylglycerol represents the major acylglycerol but other acylglycerol contents are negligible. Free cholesterol is also present in appreciable amounts. The low content of phosphatidylethanolamine may be characteristic of locust DGLP. Of interest is the presence of considerable amounts

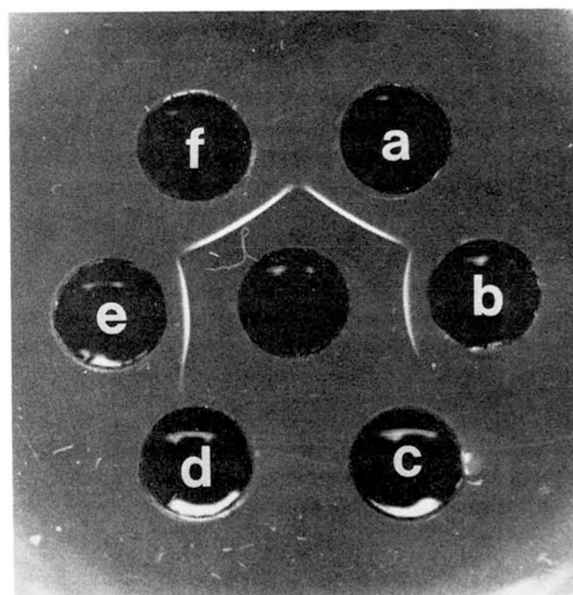

Fig. 3. Ouchterlony's double diffusion test. Center well contains 200 μg of anti-locust DGLP IgG. Well a, 25 μg male locust DGLP; well b, 25 μg male locust hemolymph; well c, 25 μg cockroach DGLP; well d, 25 μg silkworm DGLP; well e, 25 μg female locust hemolymph; well f, 25 μg female locust DGLP. The cockroach DGLP was prepared from hemolymph of male American cockroach (6). The silkworm DGLP was prepared from pupal hemolymph of *Philosamia* silkworm according to the method described in an early communication (5), but the step of precipitation by ammonium sulfate was omitted.

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TABLE 1. Summary of purification of DGLP from prelabeled hemolymph of the locust

Step or Fraction	Protein	Radioactivity of Diacylglycerol	Specific Radioactivity	Recovery of DGLP
	mg	*dpm*	*dpm/mg protein*	%
1. Original labeled hemolymph	44.5 (47.1)	388,000 (138,880)	8,720 (2,950)	100 (100)
2. Before adding eight volumes of distilled water	41.0 (45.5)	335,800 (130,620)	8,190 (2,870)	87 (94)
3. Precipitate after adding distilled water	13.3 (14.8)	305,800 (123,150)	23,000 (8,320)	79 (89)
4. DGLP (fraction A)	11.0 (9.7)	269,400 (102,750)	24,500 (10,590)	69 (74)

The prelabeled hemolymph was collected and pooled from ten male or six female locusts that had been injected with [1-^{14}C]palmitic acid (approximately 1 × 10^6 cpm/animal) 4 hr before the collection of hemolymph. The data from female locusts are given in parentheses. Protein was determined by the method of Lowry et al. (22).

of hydrocarbon in locust DGLP as well as in cockroach DGLP. Analytical data of the hydrocarbon fraction by GLC are given in **Fig. 5** and demonstrate that the fraction comprises a number of *n*-alkanes and methylalkanes ranging from C_{25} to C_{38} in chain length, but no unsaturated compounds were detected. These data display a close similarity to those reported for cuticular wax of locust by Lockey (21), who demonstrated 16 compounds of *n*-alkanes and methylalkanes ranging from C_{25} to C_{37}, but no unsaturated hydrocarbons.

Table 4 shows the amino acid composition of locust DGLP with the corresponding data from cockroach (6) and silkworm (5) DGLPs for comparison. The three DGLP molecules appear similar, being rich in asparatic acid and glutamic acid and extremely low in methionine.

GLC analysis revealed that the locust DGLP contains 3.0% mannose based on the protein amount determined by the method of Lowry et al. (22), which is significantly higher than that (0.9%) reported for cockroach DGLP (6). No other sugars were detected in appreciable amounts (GLC data not shown). The presence of glucosamine was also demonstrated by GLC, and its content, determined by the method of Blix (14), was 0.5%.

Subunit structure of locust DGLP

The subunit structure of the apoprotein of locust DGLP was analyzed by SDS-polyacrylamide gel electrophoresis of the delipidated DGLP. The delipidated DGLP from the American cockroach was subjected to the same electrophoresis for comparison. The electropherograms illustrated in **Fig. 6** (a, b, c) clearly demonstrate that the apoprotein consists of two non-identical subunits, heavy chain and light

TABLE 2. Distribution of radioactivity in lipids associated with DGLP purified from male or female locusts

Lipid Class	Male		Female	
	Radioactivity	Percentage	Radioactivity	Percentage
	dpm		*dpm*	
Total lipids[a]	93,400	100	78,090	100
1. Hydrocarbon	3,380	3.6	2,800	3.6
2. Cholesteryl ester	280	0.3	460	0.6
3. Triacylglycerol	250	0.3	240	0.3
4. Diacylglycerol	84,650	90.6	72,830	93.3
5. Monoacylglycerol[b]	2,180	2.3	1,020	1.3
6. Free fatty acid	1,330	1.4	980	1.3
7. Sum of 1–6	92,060	98.5	77,550	99.3

[a] Lipid fraction before separation by Florisil column chromatography.
[b] Radioactivity recovered in this fraction is partly due to contamination with diacylglycerol.
DGLP was purified from the pooled hemolymph of six males or five females that received 1 × 10^6 cpm [1-^{14}C]palmitic acid/animal by injection 4 hr before the collection of hemolymph.

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chain, and that the locust DGLP is not distinguishable from the cockroach DGLP in terms of subunit structure. Accordingly, the molecular weights of the two chains should be the same as those reported for cockroach (6), 250,000 for heavy chain and 85,000 for light chain, respectively. The difference between the native molecular weight (580,000) and the molecular weight of apoprotein (250,000 + 85,000) approaches the total lipid content (41%).

When the delipidated DGLP from locust or cockroach was subjected to SDS-polyacrylamide gel electrophoresis and stained for carbohydrate by the PAS technique (17), only the heavy chain was positively stained (Fig. 6d, e), indicating that sugar chains (mainly mannose) are covalently associated with heavy chain but not with light chain.

Physiological function of insect DGLP

Earlier investigations (5, 6) have demonstrated in vitro that the DGLP purified from the *Philosamia* silkworm or the American cockroach has a capacity to specifically take up diacylglycerol from the incubating fat body. The capacity of locust DGLP to serve this physiological function was tested by incubating the prelabeled fat body of the male locust in Ringer solution containing the purified DGLP, and comparing the release of diacylglycerol under these conditions with that observed when prelabeled fat body is incubated in Ringer solution only, in fresh hemolymph, or in fraction B (see Fig. 1). The results presented in **Table 5** demonstrate that the greatest release of diacylglycerol occurs in the presence of the purified DGLP. The small amount of radioactivity recovered from medium that does not contain DGLP (Ringer only or fraction B) is attributed to artificial leaking as a result of fat body breakdown during incubation. The data in Table 5 also reveal that the cockroach DGLP, the silkworm DGLP, and the locust DGLP are capable of accepting diacylglycerol from the locust fat body. This observation confirms our earlier finding (3) that the specific release of diacylglycerol occurs in any incubation combinations of fat body and hemolymph among three different insects, *Cecropia* silkworm, *Melanoplus* grasshopper, and American cockroach; in other words, the insect DGLPs exhibit no specificity in terms of their functional capacity to accept diacylglycerol from fat body.

Our previous study (6) demonstrated in vitro that cockroach DGLP has a capacity to accept diacylglycerol from the midgut as well as from the fat body. We intended to test for a similar capacity of locust DGLP in vitro. However, it was impossible to feed the adult locusts on [1-^{14}C]palmitic acid to prepare prelabeled midgut for this test. Instead, we prepared the

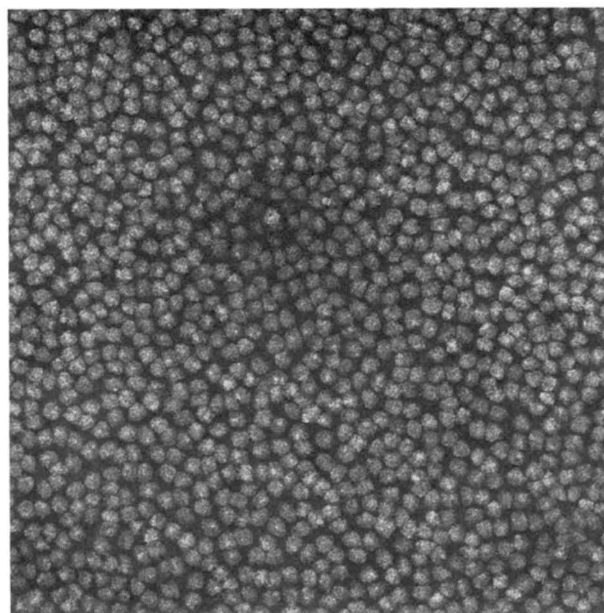

Fig. 4. Electron micrograph of locust DGLP negatively stained with uranium acetate and observed in a Hitachi 11 B electron microscope (×150,000). The measurement of molecular size was made on 100 molecules within a certain area of the electron micrograph of 550,000 magnification.

prelabeled midgut from American cockroach according to the method described in the previous paper (6), and incubated the prelabeled midgut in medium containing the cockroach DGLP, the locust DGLP, the silkworm DGLP, fraction B, or Ringer solution only. The results given in **Table 6** clearly demonstrate that the DGLPs from the three insects can take

TABLE 3. Lipid composition of DGLP from three insects

Component	% Weight[a]		
	Locust[b]	Cockroach[c]	Silkworm[d]
Protein	59	50	56
Total lipids	41	50	44
1. Hydrocarbon	21.1	28.3	1.4
2. Triacylglycerol	1.7	2.0	1.2
3. Diacylglycerol	32.8	15.2	56.3
4. Monoacylglycerol	0.0	0.0	0.0
5. Cholesterol	7.8	5.0	13.2
6. Cholesteryl ester	0.1	0.0	0.0
7. Total phospholipids	36.1	42.8	25.8
Phosphatidylcholine	(95)	(68)	(48)
Phosphatidylethanolamine	(5)	(32)	(32)
Sphingomyelin	(0)	(0)	(20)
8. Sum of 1–7	99.6	93.4	97.9

[a] Lipid fractions (1–7) are expressed as percentages of the total lipids. Each phospholipid is expressed as percentage of the total phospholipids in parentheses.
[b] DGLP purified from 50 adult locusts was combined and subjected to the analysis of the lipid composition.
[c] From data of DGLP of American cockroach (6).
[d] From data of DGLP (DGLP-I) of *Philosamia* silkworm (5), with a slight modification.

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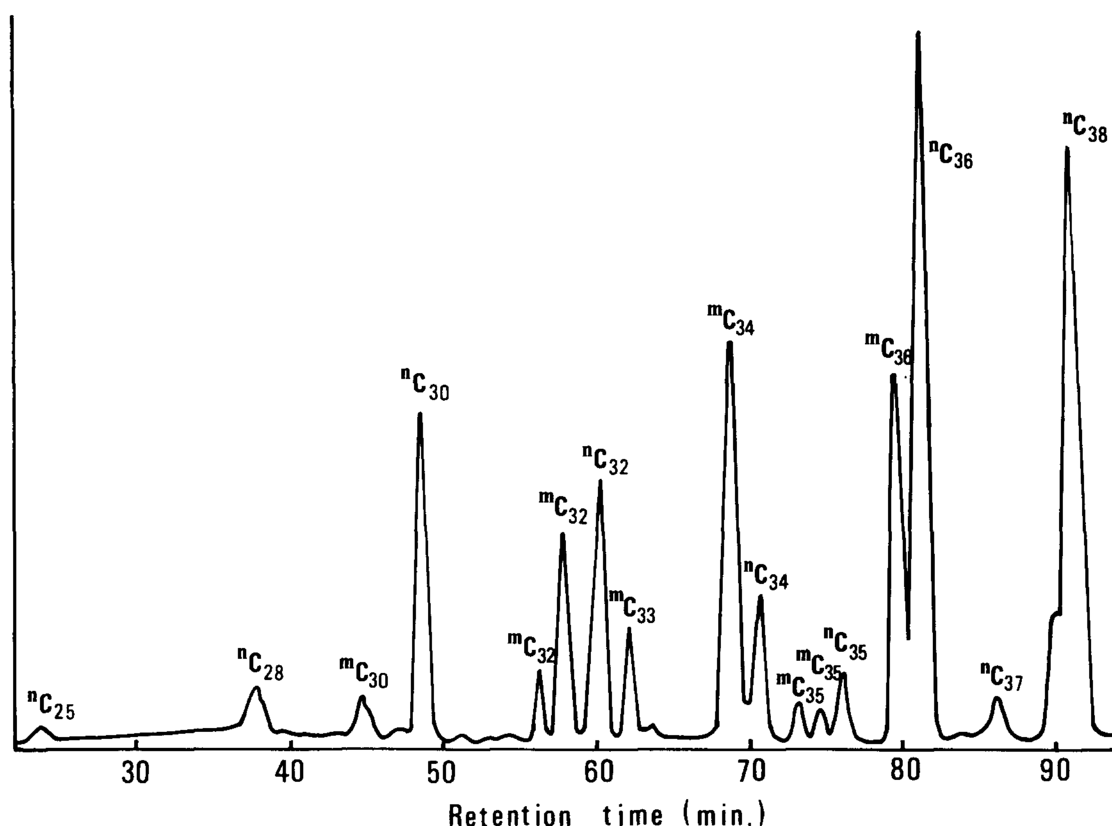

Fig. 5. Gas–liquid chromatogram of hydrocarbons of locust DGLP. A glass column (3 m × 3 mm) containing 1.5% OV-1 on Chromosorb W was run with N_2 as carrier gas, and programmed from 200°C to 320°C at 1°C/min. Each peak was further analyzed by GLC–MS (data not shown). nC, *n*-alkane; mC, methylalkane. The position of methyl branch was not determined. No unsaturated compounds were detected.

up diacylglycerol from the cockroach midgut at almost equal level, suggesting again lack of specificity in terms of this physiological function.

TABLE 4. Amino acid compositions of three insect DGLPs

Amino Acids	Recovered Amino Acids		
	Locust	Cockroach[a]	Silkworm[b]
		mol/1000 mol	
Asp	114	110	126
Thr	60	66	49
Ser	71	69	69
Glu	112	108	104
Pro	38	38	47
Gly	63	64	67
Ala	72	68	63
Val	84	84	74
Met	2	3	5
Ile	42	41	58
Leu	106	107	90
Tyr	27	30	28
Phe	47	47	48
His	37	39	28
Lys	92	93	107
Arg	26	27	37
Cys	7	6	

[a] From data of DGLP of American cockroach (6).
[b] From data of DGLP (DGLP-I) of *Philosamia* silkworm (5).

The presence of a significant amount of cholesterol in DGLP (Table 3) suggests that dietary cholesterol is absorbed from the midgut by this lipoprotein. In order to test this possibility, the following experiments were carried out. American cockroaches were fed on [4-^{14}C]cholesterol, and 48 hr after commencement of feeding, the hemolymph was collected and DGLP was purified. A typical elution profile on DEAE-cellulose column chromatography is illustrated in **Fig. 7** and demonstrates that most radioactivity is associated with the primary fraction (fraction A), exhibiting an essential similarity to the elution profile given in Fig. 1. The total radioactivity recovered in fraction A (DGLP) was 19,900 dpm, and the previous report (6) showed that the recovery of cockroach DGLP is 33% at this stage of purification. Therefore, the total radioactivity in originally pooled hemolymph is calculated to be 60,300 dpm (19,900 × 100/33), which is nearly equal to the radioactivity (61,450 dpm) actually found in the original hemolymph. This indicates that practically all the labeled cholesterol present in hemolymph is associated with the DGLP molecule. We intended to carry out further experiments in vitro to test if insect DGLPs can accept cholesterol from prelabeled midgut. Unfortunately,

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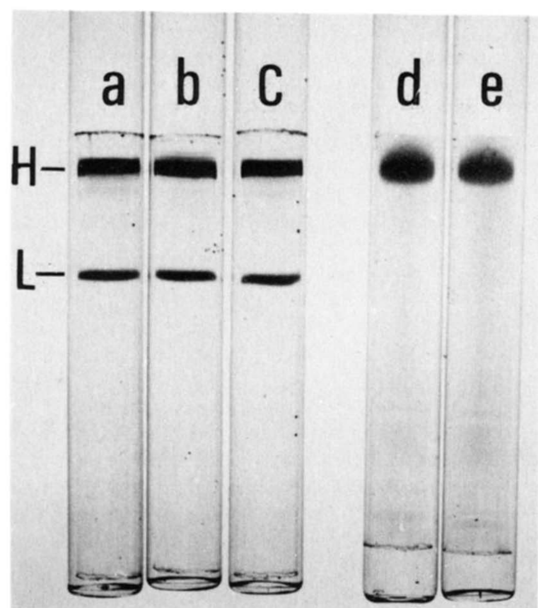

Fig. 6. SDS-polyacrylamide gel electrophoreses of locust DGLP and cockroach DGLP: a, locust DGLP; b, cockroach DGLP; c, the mixture of locust and cockroach DGLPs; d, locust DGLP; e, cockroach DGLP. The (a), (b), and (c) gels were stained for protein with Coomassie blue, and the (d) and (e) gels were stained for carbohydrate with the PAS method (this method caused a slight shortening of the gels).

the absorption of cholesterol, unlike diacylglycerol, by midgut is slow, and the difficulty of maintaining dissected midgut during a prolonged incubation period prevented the completion of this experiment.

DISCUSSION

The locust DGLP has been isolated and purified by several investigators (7–9). They have used rather complicated techniques including a precipitation by cold ethanol (7), density gradient centrifugation, repeated gel filtrations on column chromatography, or affinity column chromatography (8, 9), but satisfactory evidence to demonstrate the homogeneity of the DGLP preparation was not provided. By comparison, the method described in this report is extremely rapid and efficient in terms of the purity and yield (more than 70%) of the DGLP preparation. This method is applicable to both male and female insects, as reported for the American cockroaches (6), and the total purification procedure takes only 2 or 2.5 hr, which helps to avoid the possible denaturation of the preparation that may occur during the purification procedure.

The locust DGLP appears basically to resemble silkworm DGLP and particularly cockroach DGLP in molecular size, shape, and weight, composition of lipid, amino acid, and sugar, and the subunit structure. However, anti-locust DGLP IgG cross-reacts neither with cockroach DGLP nor with silkworm DGLP (Fig. 3), indicating high species specificity-immunological. The locust DGLP is not distinguishable from cockroach DGLP in subunit structure; the apoprotein comprises two non-identical subunits, heavy chain (mol wt 250,000) and light chain (mol

TABLE 5. Assay of diacylglycerol uptake by DGLP from prelabeled fat body of male locust

Exp. No.	Incubation Medium	Protein Amount Used for Assay	[^{14}C]Diacylglycerol Released into Incubation Medium
		mg	*dpm*
1.	Ringer solution[a]	0	10,900[b]
	Male locust fresh hemolymph	0.4	42,530
	Locust DGLP	0.4	89,770
	Locust fraction B (from male)	0.4	12,710
2.	Ringer solution	0	6,460
	Female locust fresh hemolymph	0.4	29,970
	Locust DGLP	0.4	52,710
	Cockroach DGLP	0.4	53,700
	Locust fraction B (from female)	0.4	7,050
3.	Ringer solution	0	12,960
	Locust DGLP	0.6	194,500
	Cockroach DGLP	0.6	126,400
	Silkworm DGLP	0.6	125,800

[a] 150 mM KCl, 20 mM NaCl, 5 mM $CaCl_2$ in 10 mM Tris-HCl buffer, pH 7.2.
[b] Release into a Ringer solution represents only "artificial leaking" due to disintegration of prelabeled fat body during incubation (5).

Experiment 1, 100 mg of prelabeled fat body containing 199,900 dpm [^{14}C]diacylglycerol was used for each incubation; Experiment 2, 90 mg of prelabeled fat body containing 164,000 dpm [^{14}C]diacylglycerol; Experiment 3, 100 mg of prelabeled fat body containing 608,800 dpm [^{14}C]diacylglycerol. Incubation time, 60 min at 25°C. The final volume of the incubation medium, 1 ml. For details of assay method, see the earlier papers (3, 5).

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TABLE 6. Assay of diacylglycerol uptake by DGLP from prelabeled midgut of American cockroach

Exp. No.	Incubation Medium	Protein Amount Used for Assay	[^{14}C]Diacylglycerol Released into Incubation Medium
		mg	*dpm*
1.	Ringer solution	0	3,020
	Cockroach DGLP	0.8	14,640
	Locust DGLP	0.8	13,890
	Silkworm DGLP	0.8	10,640
	Cockroach fraction B (female)	0.8	2,770
2.	Ringer solution	0	2,200
	Cockroach DGLP	1.0	13,290
	Locust DGLP	1.0	14,120
	Silkworm DGLP	1.0	9,330
	Locust fraction B (female)	1.0	1,980

The prelabeled midguts were prepared from female American cockroaches that had been fed on about 2×10^6 cpm [1-^{14}C]palmitic acid/animal 2 hr before the dissection of midgut. Incubation time was 90 min at 25°C. For details of assay method including the preparation of prelabeled midgut see the previous paper (6). [^{14}C]-labeled free fatty acid released into incubation media was negligible, indicating that no artificial leaking of gut content occurred during incubation.

wt 85,000). A similar observation has been reported for DGLP from tobacco hornworm (23). However, Gellissen and Emmerich (9) have recently reported that locust DGLP consists of an identical subunit of 85,000 molecular weight, although they have observed heavier chain (over 200,000) in some electrophoreses. They have claimed that the heavy chain observed may be an artificially aggregated product of the 85,000 unit. This, however, seems unlikely, because the present study demonstrates that carbohydrates are associated only with heavy chain but not with light chain (Fig. 6); if the heavy chain were merely an aggregate product, both chains would be stained by the PAS staining method. The data suggest that a similar subunit structure is a general feature of insect DGLP as pointed out in a previous paper (6).

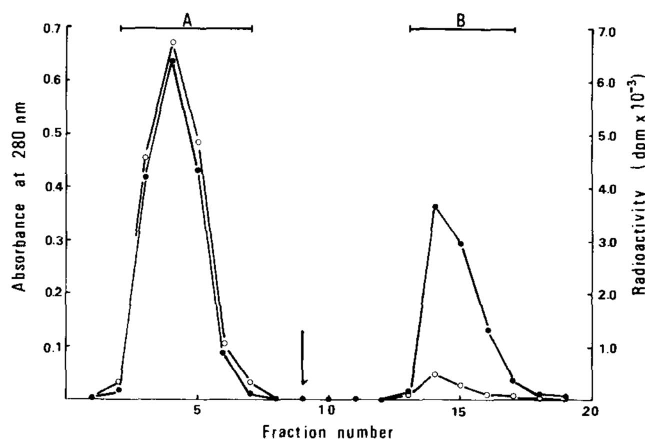

Fig. 7. DEAE-cellulose column chromatography of the fraction precipitated under low ionic concentration. Original labeled hemolymph was collected and pooled from ten male American cockroaches 48 hr after consuming [4-^{14}C]cholesterol (approximately 1×10^6 cpm/animal). The Florisil column chromatography of fraction A (DGLP) demonstrated that the radioactivity recovered in this fraction was entirely attributed to [^{14}C]cholesterol but no radioactivity was detected in cholesteryl ester fraction. Other explanations as in Fig. 1.

The present study confirms our previous observation that DGLP serves as the primary vehicle of diacylglycerol transport not only from the storage site of fat body but also from the absorption site of midgut, and, in addition, demonstrates that insect DGLPs have no species specificity in terms of the above function (Tables 5 and 6).

This study also reveals that locust DGLP and cockroach DGLP contain a considerable amount of hydrocarbons (Table 3). We described in the previous paper (6) that the hydrocarbon compounds associated with cockroach DGLP and those found as cuticle wax of the cockroach are almost the same. This is also true for the locust; GLC patterns of the DGLP hydrocarbon are very similar to those reported for the cuticular wax by Lockey (21). It is likely, therefore, that insect DGLP also serves as a carrier to transport hydrocarbons from the site of synthesis, presumably oenocytes (24), to the site of deposition (cuticle). In fact, it has recently been demonstrated in our laboratory that when the cockroach DGLP is incubated with cockroach integument (including oenocytes) prelabeled with [1-^{14}C]acetate, labeled hydrocarbons are rapidly released into incubation medium and are associated with the DGLP.[2]

The DGLPs isolated from different species contain appreciable amounts of free cholesterol (Table 3) and

[2] Katase, H., and H. Chino. Unpublished results.

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it is possible that DGLP also functions to carry cholesterol. Insects, unlike mammals, are unable to synthesize sterols from acetate and are entirely dependent upon a dietary source of sterols. Therefore, the transport of absorbed cholesterol to sites of utilization is of considerable importance for the insects. Chino and Gilbert (25) demonstrated that DGLP from *Philosamia* silkworm can take up cholesterol from the incubating larval midgut and, therefore, it serves as a cholesterol-carrier. In this study, we demonstrated that when American cockroaches are fed [4-^{14}C]-cholesterol, practically all the labeled cholesterol absorbed by the digestive tract is associated with DGLP (Fig. 7 and the text). This observation supports the suggestion that the cockroach DGLP also functions as a cholesterol-carrier, although it was not possible to perform the definite experiment for demonstrating this function in vitro.

The above observations together with accumulated knowledge on lipid transport in insects lead to the conclusion that, in many insects, DGLP serves multiple functions in transporting various lipids including diacylglycerol, cholesterol, hydrocarbon, and probably carotenoid from sites of storage, absorption, or synthesis to sites where these lipids are utilized as a metabolic fuel, precursors for triacylglycerol and phospholipid synthesis, or structural components of cell membrane and cuticle.

The physiological significance of insect DGLP may become clearer by comparing the functions of DGLP with those reported for mammalian plasma lipoproteins. In mammals, several lipoproteins, including chylomicrons, very low density lipoprotein, and low density lipoprotein, are involved in transporting such lipids as triacylglycerol and cholesteryl ester from intestine or liver. These lipoprotein molecules are originally formed in the cells of these tissues and are released into lymph or blood. The triacylglycerol associated with these molecules is hydrolyzed by a lipoprotein lipase located in the capillary wall, and the resulting remnant is ultimately broken into small fragments in the liver or extrahepatic tissues. By contrast, insect DGLP is primarily formed in the fat body, released into hemolymph and loads various lipids, probably on the surface of different tissues, and unloads these lipids at the site of utilization, although the mechanism of loading and unloading lipids is unknown; thus, the same molecule can perform this function repeatedly. This proposal is supported by study (26) with *Philosamia* silkworms in which DGLP isolated from eggs has been shown to contain much less diacylglycerol than DGLP isolated

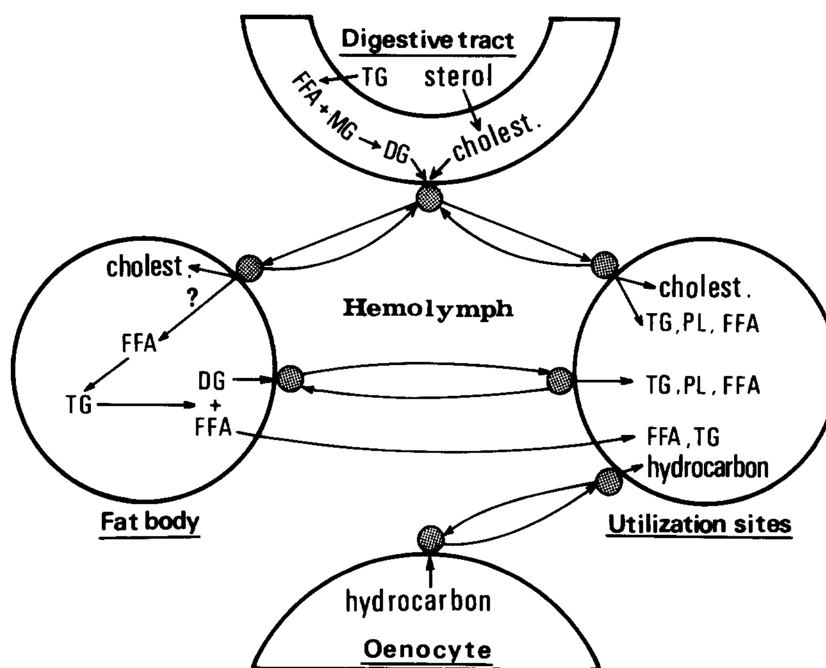

Fig. 8. A scheme proposed for lipid transport in insects. Small circles, DGLP molecule; TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; FFA, free fatty acid; PL, phospholipid. The release of diacylglycerol by the tissue is known to be an active process requiring ATP whereas the release of free fatty acid is a simple diffusion (3, 5). It is assumed that under natural conditions, the absorption and transport of diacylglycerol at the digestive tract occurs mainly in polyphagous insects such as the cockroach, and that the stored triacylglycerol in fat body is derived from carbohydrate in such monophagous insects as the silkworms that feed only on plant leaves during the larval stage.

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from hemolymph. This observation suggests that DGLP unloads diacylglycerol in the ovary during oogenesis, but it was demonstrated in vitro that the "empty" molecule retains its capacity to accept diacylglycerol from incubating fat body (26). Thus, from the viewpoint of physiological function, insect DGLP is a true carrier molecule and acts as a reusable shuttle, unlike most mammalian lipoproteins which appear to function only once. It is apparent from the above discussion that the mechanism of lipid transport in insects is basically different from that of the mammals, and this may reflect the difference in the circulation system between insects and mammals with an open system in the former and a closed system in the latter.

Finally, the proposed scheme of lipid transport in insects is illustrated in **Fig. 8** and indicates that the insect DGLP serves multiple functions as a true carrier molecule.

The authors wish to thank Dr. K. Takahashi, Department of Animal Science of this University, for electron microscopy, Dr. S. Gasa, School of Medicine of this University, for the GLC–MS analyses, and Mrs. Y. Abe for her assistance throughout the experiments. Thanks also due to Dr. R. G. H. Downer, University of Waterloo, Canada, for reading the manuscript. This work was partly supported by a research grant (448020) from the Japanese Ministry of Education.

Manuscript received 21 January 1981 and in revised form 22 April 1981.

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