Adipokinetic hormone-induced lipolysis in the fat body of an insect, *Manduca sexta:* synthesis of *sn*-1,2-diacylglycerols

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Abstract The pathway for the adipokinetic hormone-stimulated synthesis of *sn*-1,2-diacylglycerols in the adult *Manduca sexta* fat body was studied. Adult fat body lipids were labeled by feeding 5th instar larvae either with [9,10(n)-^{3}H]oleic acid or [1(3)-^{3}H]glycerol and after 32 days insects at the adult stage were used. This long-term prelabeling led to labeled fat body acylglycerols in which triacylglycerols comprised the main radioactive lipid component (95.5%), regardless of the radiolabeled compound used. Because the distribution of radioactivity among the lipid classes was very close to the mass distribution of the fat body lipid subspecies, it was concluded that homogeneous labeling of fat body lipids was obtained. After adipokinetic hormone treatment, an accumulation of radioactivity in the *sn*-1,2-diacylglycerol fraction was the only significant change found in the distribution of radioactivity among fat body lipids. The size of diacylglycerol pool increased 280% 60 min after adipokinetic hormone stimulation, whereas the fatty acid, monoacylglycerol and phosphatidic acid pool sizees remained constant. These results support the hypothesis that adipokinetic hormone-stimulated synthesis of *sn*-1,2-diacylglycerol in the fat body involves stereospecific hydrolysis of the triacylglycerol stores.—**Arrese, E. L., and M. A. Wells.** Adipokinetic hormone-induced lipolysis in the fat body of an insect, *Manduca sexta:* synthesis of *sn*-1,2-diacylglycerols. *J. Lipid Res.* 1997. **38:** 68–76.

Supplementary key words lipid mobilization • fat body • AKH • diacylglycerol • *Manduca sexta*

The insect fat body, which combines many of the properties and functions of vertebrate liver and adipose tissue, is the principal site in insects for the storage of lipids (1), and triacylglycerols (TG) constitute the main lipid storage form, representing about 90% of the total fat body lipid (2). The content of TG in the fat body is influenced by several factors, including development stage, nutritional state, and migratory flight (3). In the tobacco hornworm, *Manduca sexta,* which is widely used as a model insect, the maximum content of fat body TG occurs at the end of larval development, as a consequence of the accumulation of reserves during larval feeding (4). Afterwards, as a result of lipolysis and the fatty acid oxidation required to sustain energy metabolism during the subsequent non-feeding pupal and adult periods, the TG stores decline (4, 5).

Two factors of cephalic origin have been shown to activate lipolysis in the fat body: adipokinetic hormone (AKH) and octopamine. AKH, a peptide that is released from the corpora cardiaca into the hemolymph during flight in many insects, greatly stimulates the secretion of *sn*-1,2-diacylglycerol from the fat body (6). Octopamine, a monohydroxyphenolic analogue of noradrenaline, whose secretion is stimulated by stress, was shown to induce lipid mobilization in *Locusta migratoria* (7) and *Acheta domesticus* (8). Adult *M. sexta* show only a moderate response to octopamine compared to AKH (E. L. Arrese and M. A. Wells, unpublished results). Starvation also stimulates lipid mobilization by an uncharacterized mechanism that is independent of AKH (5).

Unlike vertebrates, where the fatty acids in the stored TG are mobilized as free fatty acids (FFA), in insects, most, if not all, fatty acids are released from the fat body as *sn*-1,2-diacylglycerols (DG) (3). The DGs are transported from the fat body to sites of utilization by lipophorin, the major lipoprotein present in the hemolymph of most insects (9).

The mechanisms of stereospecific synthesis and secretion of *sn*-1,2-DG are unknown. Three different pathways for the formation of DG have been proposed: *1*) the hydrolysis of TG into *sn*-2-monoacylglycerol

Abbreviations: TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; FFA, free fatty acids; PL, phospholipid; PA, phosphatidic acid; AKH, adipokinetic hormone; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; MGAT, monoacylglycerol-acyltransferase

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(MG) followed by stereospecific acylation of *sn*-2-MG (10–12); *2*) de novo synthesis of DG from *sn*-glycerol-3 phosphate via phosphatidic acid (PA) using the fatty acids produced by TG hydrolysis (13); and *3*) the stereospecific hydrolysis of TG into *sn*-1,2-DG (14, 15).

In the first step of lipid mobilization, TG must be hydrolyzed by the action of a lipase. Several investigators have studied insect fat body lipase activity (16) and recently we purified a TG-lipase from *M. sexta* fat body (13). Like vertebrate hormone-sensitive lipase, which catalyzes the rate-limiting step in mobilization of adipose tissue fatty acids (17), the *M. sexta* fat body TG-lipase is a phosphorylatable enzyme (13). In vitro, TG hydrolysis catalyzed by the TG-lipase led to the accumulation of *sn*-2-MG, which suggested that the monoacylglycerol pathway might be the major route for synthesis of *sn*-1,2-DG in *M. sexta* fat body. Such a pathway requires the activity of a monoacylglycerol-acyltransferase (MGAT). This enzyme is present in *M. sexta* fat body microsomes (18); however, we found no effect of AKH on MGAT activity, suggesting that AKH does not stimulate the synthesis of *sn*-1,2-DG through the activation of MGAT.

The experiments described below were performed to gain more information about this fundamental metabolic pathway. We have studied the pathway for the synthesis of *sn*-1,2-DG from stored TG in the adult *M. sexta* fat body when lipid mobilization is stimulated by AKH. Our data indicated that the most likely origin of the *sn*-1,2-DG is via stereospecific hydrolysis of TG.

MATERIALS AND METHODS

Materials

Insects from a colony maintained in this laboratory were used (19). Adults were kept without food at 25°C. [9,10(n)-^{3}H]oleic acid was purchased from New England Nuclear (Boston, MA) and [1(3)-^{3}H]glycerol was purchased from Amersham (Arlington Heights, IL). *M. Sexta* AKH was purchased from Peninsula Laboratories (Belmont, CA). Phospholipase A_2 (porcine pancreas) was purchased from Sigma Co. (St. Louis, MO). Silica gel G plates were obtained from J. T. Baker (Phillipsburg, NJ). All other chemicals were of analytical grade.

Experimental insects

In order to eliminate the release of endogenous brain components in response to handling, the head of the experimental animal was removed with scissors and the wound was sealed with petroleum jelly (20). After 24 h, decapitated insects were injected with 20 µl of an aqueous trehalose solution (0.65 g/ml), which reduces the endogenous fat body TG-lipase activity to a minimum value (20). Two h later the insects were injected with AKH (100 pmol), dissolved in 5 mM NaH_2PO_4 buffer, pH 6.5 containing 0.1 M KCl, 18 mM $MgCl_2$, 4 mM NaCl, and 1 mg/ml of defatted bovine serum albumin. Fat body and hemolymph were taken at various times after AKH injection.

Long-term labeling of fat body lipids

During the fifth larval instar, insects were fed 50 µCi of either [9,10(n)-^{3}H]oleic acid or 100 µCi [1(3)-^{3}H]glycerol. The desired amount of labeled fatty acid or glycerol was dissolved in a small volume of ethanol, and a 10-µl aliquot was spread onto a small piece of flat insect diet which was fed to the insects individually. The insects were then allowed to complete normal development, which required about 32 days, and radioactively labeled adults, 2 or 3 days after emergence, were used. Two independent experiments were carried out using three insects for each time point. The samples from each insect were individually analyzed. Because both experiments gave similar results, the experimental data were combined to obtain the average and SEM values.

Sample preparation and lipid extraction

Hemolymph was collected by the "flushing out method" (21), using 30 mM KH_2PO_4 buffer, pH 7.0, containing 2 mM EDTA. After hemolymph collection, the fat body was dissected rapidly and rinsed with the same buffer, which was chilled on ice. Hemolymph and fat body lipids were individually extracted with chloroform–methanol (22). A small aliquot of the lipid extract was used for determination of the total lipid radioactivity by liquid scintillation counting. The amount of radioactivity present in the fat body and hemolymph of insects that were fed radioactive oleic acid ranged between 4.5 and 6 µCi and 0.5 and 0.6 µCi per insect, respectively. The amount of radioactivity present in the fat body and hemolymph of insects that were fed radioactive glycerol ranged between 1.7 and 2.6 µCi and 0.3 and 0.7 µCi per insect, respectively.

Lipid separation

The lipids in the extracts were separated by thin-layer chromatography (TLC) on silica gel G plates using hexane–ethyl ether–formic acid 70:30:3 (v/v/v) as the developing solvent. The MG, DG, FFA, and TG fractions, along with material that remained at the origin, the phospholipid fraction (PL), were visualized with I_2 vapor, scraped from the plate, and the radioactivity was counted by liquid scintillation counting.

The separation and identification of PA was per-

formed by TLC using the method of Possmayer et al. (23). Briefly, the material that remained at the origin of the TLC plate after the above chromatography was eluted from the plate with chloroform–methanol–acetic acid–water 50:39:1:10 (v/v/v), and the PA was separated from the other PL by chromatography on silica gel 60 plates that were impregnated with a 0.054 M oxalic acid solution, pH 7.2, and activated at 100°C for 15 min (24). The developing system was chloroform–methanol–acetic acid–water 55:43:3:4 (v/v/v). The PA fraction, detected with I_2 vapor, was scraped from the plate and the rest of the lane, which contained the remaining PL, was also scraped from the plate and combined as a single fraction. The radioactivity of the PA fraction and the rest of the lane was determined as indicated above, and the percentage of total PA radioactivity present in the PL fraction was calculated. Results were expressed as percent of total radioactivity (mean ± SEM, n = 3–5).

Fat body lipid composition

Total lipid extracts of fat body were obtained as described above and the lipid fractions (PL, MG, DG, FA, and TG) were also separated by TLC as mentioned previously. The lipid classes were scraped from the plate and lipids were eluted from the silica with diethyl ether (25) except for PL which were eluted using a mixture of chloroform–methanol–acetic acid–water 55:43:3:4 (v/v/v) (24). MG, DG, FFA, and TG samples were transferred to a glass conical tube containing appropriate amounts of monoheptadecanoyl-glycerol, diheptadecanoyl-glycerol, heptadecanoic acid, and triheptadecanoyl-glycerol, which served as internal standards for the quantitation of MG, DG, FA, and TG, respectively. The lipid samples were transesterified with HCl–methanol. Fatty acid methyl esters were chromatographed in a Shimadzu GC mini-2 GLC on PT 10% Silar-10C (Alltech, Deerfield, Ill) packed columns.

The amount of phospholipid was determined by measuring the mass of phosphorus present in the PL fraction (26). A blank sample that was obtained by eluting a similar area of silica gel was analyzed simultaneously. Results were expressed as percent of total lipid class (mean ± SEM, n = 3).

The specific radioactivity of the fat body DG pool at 0 and 60 min after AKH injection was calculated as the ratio of the percent of total radioactivity present as DG/the percent of total lipid mass present as DG. The mass of fat body DG was determined by gas chromatography, as indicated above.

Stereospecific analysis of 1,2(2,3)-DG

The stereochemistry of the *sn*-1,2(2,3)-DGs from the fat body and hemolymph of insects labeled with radioactive glycerol was determined by the method of Brockerhoff (27). Control insects and insects 60 min after AKH (100 pmol) injection were used. Lipids were separated by TLC on silica gel G plates impregnated with 1.2% boric acid, using chloroform–acetone 96:4 (v/v) as the developing solvent (24). Lipids were visualized using iodine vapor and spots corresponding to *sn*-1,2(2,3)-DG were scraped from the plate and eluted from the silica with diethyl ether (25). The DGs were converted to a mixture of D- and L-phosphatidyl phenols that were isolated as the triethylammonium salt (27). The D,L-phosphatidyl phenols were dissolved in 1 ml diethyl ether and mixed with 1 ml Tris-HCl buffer (0.1 M, pH 8.6) containing 6 mM $CaCl_2$, and two aliquots of 0.5 ml were removed. To one aliquot was added 250 units of phospholipase A_2 and then both reaction mixtures were shaken continuously for 3 h at 37°C. The products were separated on silica gel G plates with the solvent mixture chloroform–methanol–3% aqueous ammonia 63:30:7 (v/v/v). The untreated D,L-phosphatidyl phenols gave one spot on TLC, whereas the treated fraction gave two spots corresponding to the D-phosphatidyl phenol and the L-lysophosphatidyl phenol. The radioactivity of the scraped spots was determined. Duplicate determinations were carried out.

Positional distribution of radiolabeled fatty acids in acylglycerols

TG and DG were separated by TLC as indicated above, and the fractions were scraped from the plate and the lipids were eluted from the silica gel as previously indicated. The lipid samples were used to analyze the positional distribution of the radioactively labeled fatty acids according to Brockman (28). Briefly, lipids were digested with pancreatic lipase, the reaction was terminated by the addition of 2.5 ml of chloroform–methanol–benzene 2:2.4:1 (v/v/v) and 150 µl of 1 N HCl, and the organic phase was concentrated to dryness. The residue was dissolved in 20 µl of chloroform–methanol 2:1 (v/v) and applied to a silica gel G TLC impregnated with 1.2% boric, and separated as described above. Fractions corresponding to 1-MG, 2-MG, 1,2(2,3)-DG, 1,3-DG, FFA, and TG were scraped from the plate and radioactivity was counted as indicated above.

Distribution of the radiolabel between the glycerol backbone and fatty acids in acylglycerols

Fat body lipid extracts from insects that were fed with radioactive glycerol were separated by TLC as indicated above. Fractions were scraped from the plate, lipids were eluted from the silica gel as previously indicated and concentrated to dryness under a stream of nitrogen. KOH, 200 µl of a 0.5 N ethanolic solution, was

added and the contents were incubated at 60°C for 1 h in a water bath. After the addition of 20 µl of 12 N HCl, fatty acids and glycerol were separated by adding 5 ml of chloroform–methanol 2:1 (v/v) and 1.05 ml of 0.8% (w/v) KCl in water. The mixture was vortexed and centrifuged at 2,000 *g* at room temperature. The organic and aqueous phases were separated, washed, and aliquots were taken to measure radioactivity. Determinations were done in duplicate.

RESULTS

Labeling of fat body lipids

Metabolic heterogeneity of cellular acylglycerols has been shown in vertebrate adipose tissue, where the existence of an "active" pool, containing the latest acylglycerols formed, has been reported (29–31). Preliminary data indicated the occurrence of different metabolic pools of acylglycerols in the insect fat body. Such metabolic heterogeneity would complicate interpretation of precursor–product relationships based on measuring the conversion of labeled TG to DG. Therefore, long-term labeling of fat body TG stores by feeding labeled compounds during the larval stage was investigated as a means to obtain homogeneously labeled TG stores in the fat body of of adult insects.

The distribution of total radioactivity between the fat body and the hemolymph in adult insects that were fed [^{3}H]oleic acid during the larval stage was 88.8 ± 1.2% and 11.2 ± 1.2%, respectively. About 85% of the total label in the insect was found in the TG that are stored in the fat body (**Table 1**), and TG comprised the main radioactive lipid (95.6 ± 2.2%) found in the fat body, with DG, PL, MG, and FFA accounting for 2.7 ± 0.2%, 0.82 ± 0.2%, 0.14 ± 0.05% and 0.7 ± 0.02% of the total radioactivity, respectively (**Table 2**). Analysis of the positional distribution of radiolabeled fatty acids in the fat body TG indicated that 22% of the label was incorporated into the *sn*-2-position, whereas 78% was in the *sn*-1(3)-position.

The distribution of radioactivity among the lipid classes is very close to the mass distribution of the lipid species (Table 2). Because of this and the length of time elapsed between feeding the radiolabeled lipid to larvae and the time the insects became adults, approximately 32 days, one can assume that a homogeneous distribution of radiolabel in the lipid species in the fat body has been achieved. This means that the distribution of radioactivity among the acylglycerols (TG, DG, and MG) is equivalent to the molar content of the acylglycerols in the fat body and hemolymph, and that changes in the distribution of radioactivity among the lipid subspecies that follows hormonal stimulation are a measure of the change in mass of the different lipids.

Pathway for DG synthesis stimulated by AKH

Experiments using insects labeled with radioactive fatty acids. **Figure 1** shows the effect of AKH on the distribution of radioactivity between the fat body and hemolymph. Initially, 89% of the total radioactivity was found in the fat body. A gradual increase in the proportion of radioactivity present in the hemolymph was observed after AKH stimulation. A statistically significant increase was detected 10 min after hormone injection, and after 60 min, the percentage of radioactivity in the hemolymph was increased by 100%.

Figure 2 shows the time course of the distribution of radioactivity among fat body lipids after injection of AKH. The levels of FFA, MG, and PA remained constant during the progress of the experiment. The variations in MG content detected just after hormone injection were not statistically significant. The only significant change was observed in the DG level which gradually increased from 2.7% to 6.5% 60 min after AKH stimulation. The specific radioactivity of the fat body DG pool 60 min after AKH injection, 0.94 ± 0.05 (% cpm in DG/% lipid mass as DG) was not significantly different from the value at the beginning of the experiment, 1.07 ± 0.03 (% cpm in DG/% lipid mass as DG). This results

TABLE 1. Distribution of radioactive lipids between the fat body and hemolymph in adult insects whose lipids were prelabeled during the larval stage using [9,10(n)-^{3}H]oleic acid

		Lipid Classes				
Tissue	Radioactivity	TG	DG	MG	FFA	PL
	cpm × 10^{-6}			*% cpm*		
Fat body	3.38 ± 0.30	84.4 ± 1.90	2.43 ± 0.14	0.12 ± 0.14	0.58 ± 0.01	0.73 ± 0.14
Hemolymph	0.43 ± 0.01	0.43 ± 0.24	9.86 ± 1.35	0.11 ± 0.04	0.08 ± 0.02	0.86 ± 0.17

Lipid extracts of fat body and hemolymph obtained from individual insects were analyzed for radioactivity, and aliquots were separated by TLC into component major classes. Radioactivity is expressed as cpm ± SEM (n = 3–5) per insect. Lipid components are expressed as percentage of total cpm ± SEM (n = 3–5). Total cpm represents the cpm that were recovered from the fat body plus the hemolymph per insect.

TABLE 2. Fat body lipid composition and distribution of radioactivity among fat body lipids in adult insects whose lipids were prelabeled during the larval stage using [9,10(n)-^{3}H]oleic acid or [^{3}H]glycerol

	TG	DG	MG	FFA	PL
Chemical analysis (% mass)	94.33 ± 3.78	2.61 ± 0.25	0.24 ± 0.05	0.50 ± 0.10	2.31 ± 0.04
Feeding [^{3}H]oleic (% cpm)	95.60 ± 2.10	2.80 ± 0.16	0.14 ± 0.04	0.66 ± 0.01	0.82 ± 0.16
Feeding [^{3}H]glycerol (% cpm)	95.60 ± 3.70	2.43 ± 0.12	0.04 ± 0.01	0.01 ± 0.007	2.10 ± 0.21

The results are expressed as percentage of total lipid mass (mean ± SEM, n = 3) for the chemical analysis data, and as percentage of total cpm found in the fat body lipid extract (mean ± SEM, n = 3–5) for the distribution of radioactivity.

confirms that uniform labeling of the fat body lipids was attained.

Stereospecific analysis of the fat body DG pool isolated from insects 60 min after treatment with AKH showed that *sn*-1,2-DG represented 89.8% of the total DG, while the *sn*-2,3- and *sn*-1,3-isomers represented 2.8% and 7.4%, respectively. A very similar composition was found for the DG isolated from the fat body of control insects.

As expected, the major labeled lipid released from the fat body into the hemolymph was DG, whereas hemolymph MG and FFA remained relatively constant as a percent of total hemolymph lipid (**Fig. 3**). Among the different isomers of DG, *sn*-1,2-DG represented 88.4%, while the *sn*-2,3- and 1,3-isomers represented 4.6% and 7% of the radioactive hemolymph DG, respectively. This result is consistent with previous studies in *Locusta migratoria* that showed that the fat body preferentially releases *sn*-1,2-DG (3).

The analysis of the positional distribution of the radiolabeled FFA in the hemolymph DG showed that 35% of the radiolabeled FFA was present at the *sn*-2-position.

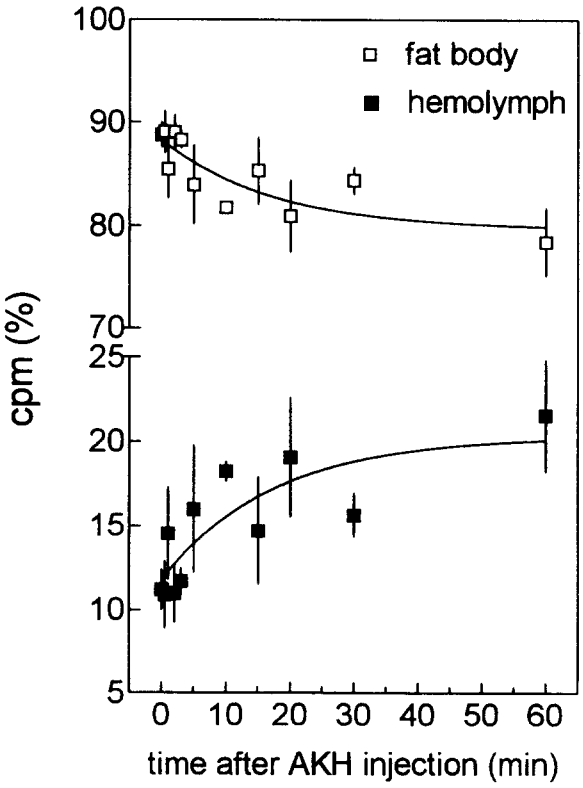

Fig. 1. Effect of AKH on the distribution of total radioactivity between the fat body and hemolymph in insects labeled with [^{3}H]oleic acid. Lipids extracts of fat body and hemolymph were analyzed for radioactivity. Results are expressed as percentage of total cpm ± SEM. Total cpm represents the cpm that were recovered from the fat body plus the hemolymph.

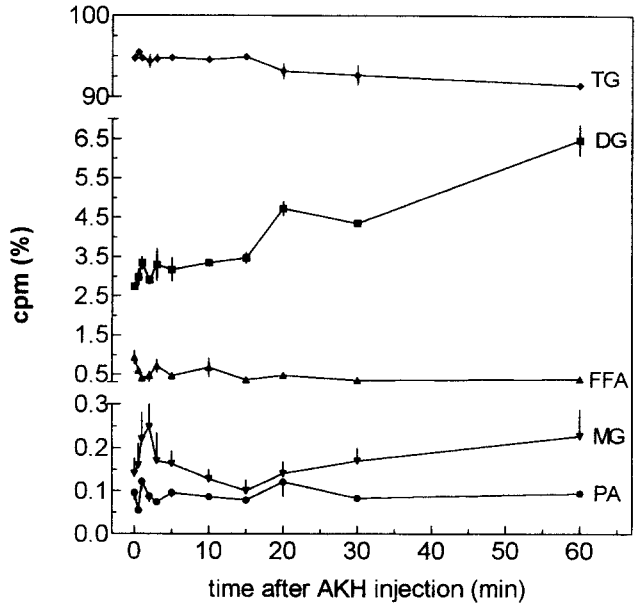

Fig. 2. Time course of the distribution of radioactivity among fat body lipids after injection of AKH in insects labeled with [^{3}H]oleic acid. Fat body lipid extracts were separated by TLC. The distribution of radioactivity was determined by liquid scintillation assay of silica gel scrapings. Results are expressed as percentage of total cpm ± SEM where the sum of cpm obtained from PA (●), MG (▼), DG (■), FFA (▲), and TG (◆) represents the 100%. The specific radioactivity of DG, expressed a % cpm in DG/% lipid mass as DG, was 1.07 ± 0.03 and 0.90 ± 0.14 at 0 and 60 min after AKH treatment, respectively. The difference between the apparent specific radioactivity at the two time points was not statistically significant.

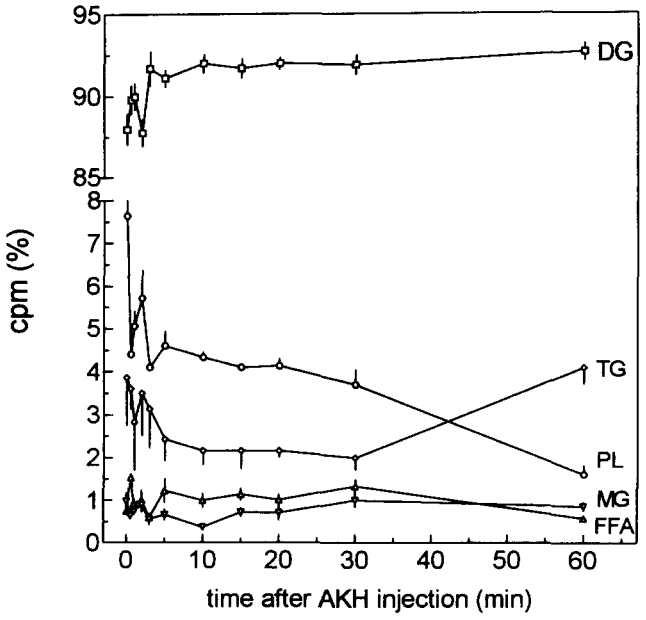

Fig. 3. Time course of the distribution of radioactivity among hemolymph lipids after AKH stimulation in insects labeled with [^{3}H]oleic acid. Hemolymph lipid extracts were separated by TLC. The distribution of radioactivity was determined by liquid scintillation assay of silica gel scrapings. Results are expressed as percentage of cpm ± SEM, where the sum of cpm obtained for PL (○), MG (▽), DG (□), FFA (△), and TG (◇) represent 100%.

The enrichment of labeled fatty acid at the *sn*-2 position of the hemolymph DG, compared to the fat body TG, is consistent with a pathway in which fat body TG are converted directly to DG by stereospecific hydrolysis. Thus, if the *sn*-3 fatty acid is lost during conversion of TG to DG, the expected amount of labeled fatty acid at the *sn*-2 position of DG, based on the fact that 22% of the labeled fatty acid was found at the *sn*-2 position of the fat body TG, would be 36%, which is the amount found.

Figure 4 shows the increase in the radioactivity of hemolymph DG, as well as the increase in the mass of DG present in the hemolymph after stimulation by AKH. It can be seen that the apparent specific activity of hemolymph DG remained constant for the duration of the experiment. This result is also consistent with the conclusion that the long-term prelabeling of the fat body lipids led to homogeneously labeled acylglycerols.

Experiments using insects labeled with radioactive glyerol. In the previous experiments the potential production of 2-MG during lipid mobilization cannot be followed due to the fact that the fat body TG was radiolabeled predominately in the FFA located at the *sn*-1-position. Because we were unable to specifically label the *sn*-2 position of the fat body TG, we labeled the glycerol backbone of the fat body TG by feeding the animals with radioactive glycerol. When insects in the 5th instar larval stage were fed radioactive [1,3-^{3}H]glycerol, fat body TG represented the main radiolabeled lipid component found in the fat body (Table 2). The distribution of radioactivity among the lipid classes was also very similar to the fat body lipid composition (Table 2). With labeled glycerol, the proportion of PL was slightly higher than found when insects were fed [^{3}H]oleic acid, whereas the amount of radioactivity found in FFA was much smaller. The analysis of the localization of the label in the TG molecule showed that 80% of the radioactivity was in the glycerol backbone and 20% in the fatty acids. Given this distribution of radioactivity, the acylglycerols can be considered to be homogeneously labeled. The time course of the distribution of glycerol radioactivity among fat body lipids after injection of AKH is shown in **Fig. 5.** A gradual increase in the DG level, which was statistically significant after 15 min, was observed. A 280% increase was detected 60 min after hormonal stimulation of lipolysis. The specific radioactivity of the fat body DG pool at 60 min after hormonal treatment was 1.30 ± 0.27 (% cpm in DG/% lipid mass as DG), which was not significantly different from the specific radioactivity at 0 time, 0.94 ± 0.05 (% cpm in DG/% lipid mass as DG). This observation is also consistent with the conclusion that long-term prelabeling of the fat body lipids led to uniformly labeled acylglycerols. These results were similar to those obtained using long-term fatty acid prelabeled insects. No significant changes were detected in the levels of MG, PA, or FFA upon hormonal stimulation. Because the level of MG remained unchanged during the progress of the experiment, the possibility that MG is an intermediate in the conversion of TG to DG can be ruled out.

DISCUSSION

Experimental animals

Although in vivo studies with intact insects might be considered the most physiologically relevant approach, the interpretation of the data is often confounded by the complexity of the system, especially by the effects of endogenous hormones, which can be released as a result of handling the insects. In vitro fat body culture might eliminate the effects of such hormones, but, for reasons that remain unclear, the mobilization of DG from the fat body does not occur with the same efficiency that occurs in vivo (32–34). Therefore, we developed a system that uses decapitated insects injected with trehalose 2 h prior to the experiment. In this system the confounding effects of endogenous hormone release

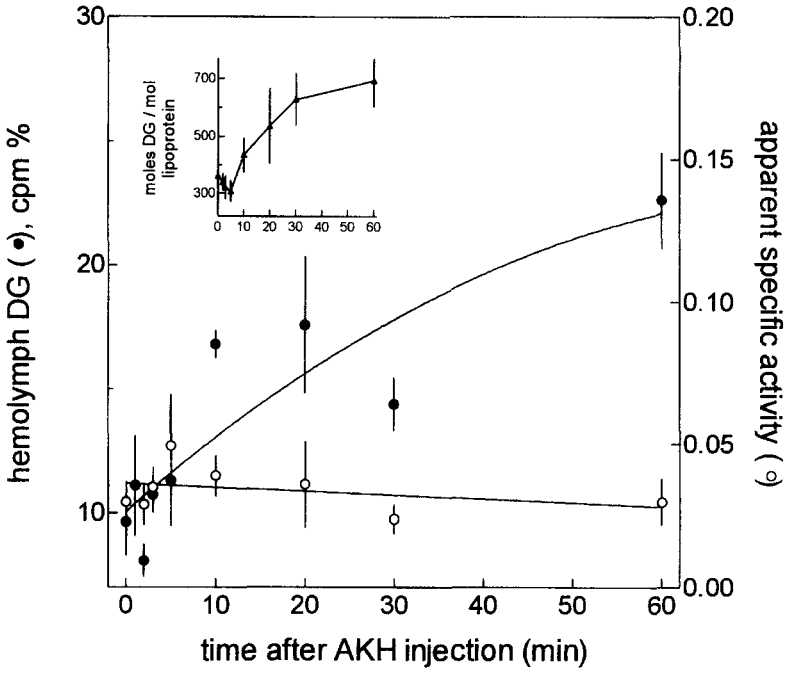

Fig. 4. Time course of the increase in the radioactivity of hemolymph DG after AKH stimulation in insects labeled with [^{3}H]oleic acid. The radioactivity of hemolymph DG is expressed as percentage of total cpm recovered from the fat body and hemolymph. The apparent specific radioactivity was calculated as the ratio between the % of cpm and the mass of hemolymph DG (mol DG/mol lipoprotein). The amount of total lipoprotein present in the hemolymph remains constant after AKH treatment.

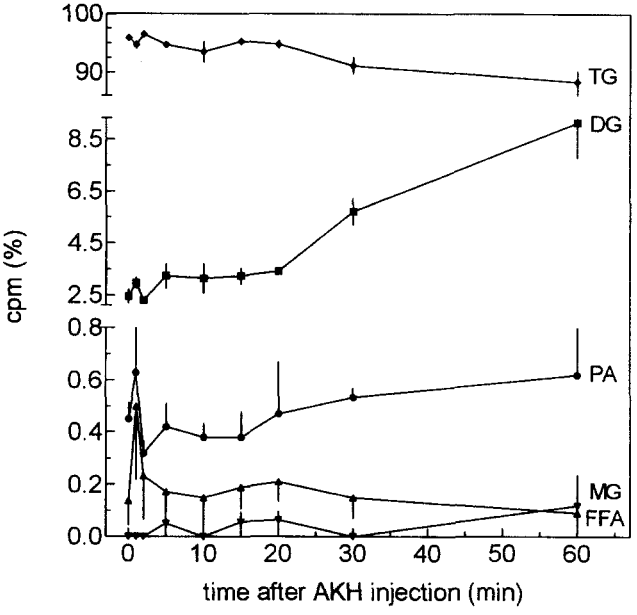

Fig. 5. Time course of the distribution of radioactivity among fat body lipids after AKH stimulation in insects labeled with [^{3}H]glycerol. Fat body lipid extracts were separated by TLC. The distribution of radioactivity was determined by liquid scintillation assay of silica gel scrapings. Results are expressed as percentage of cpm ± SEM where the sum of cpm obtained for PA (●), MG (▼), DG (■), FFA (▲), and TG (◆) represents the 100%. The apparent specific radioactivity of DG, expressed as % cpm in DG/% lipid mass as DG, was 0.94 ± 0.05 and 1.30 ± 0.27 at 0 and 60 min after AKH treatment, respectively. The difference between the apparent specific radioactivity at the two time points was not statistically significant.

from the brain in response to stress and starvation, which is the condition in which adult *M. sexta* are kept in the laboratory, were eliminated. Before injection with AKH, these experimental animals showed a low level of fat body TG-lipase activity, and after AKH injection showed activation of the lipase and a high level of lipid mobilization from the fat body (20).

Pathway for the synthesis of *sn*-1,2-DG

Under conditions of energy demand, i.e., flight in which AKH is secreted, the fat body releases *sn*-1,2-DG into the hemolymph. The pathway for its formation could involve: *1*) the acylation of *sn*-2-MG; *2*) de novo synthesis from *sn*-3-glycerophosphate; or *3*) the stereospecific hydrolysis of TG.

The MG pathway begins with the hydrolysis of TG to yield *sn*-2-MG, followed by its reacylation to *sn*-1,2-DG. The first step is catalyzed by the TG-lipase and the second by the microsomal enzyme MGAT. Our data show that the content of fat body MG, which represents about 0.2% of total lipids in the fat body, remains constant when the fat body is secreting DG into the hemolymph. Two different possibilities could explain this observation. First, the fat body MG pool does not increase during the activation of lipolysis because MG is not an intermediate of the conversion of TG to DG. Alternatively, if the acylation of MG occurs at the same rate as its formation, the level of MG would stay constant. Previously, we studied the effect of AKH on the activity of fat body MGAT by measuring the enzyme activity in microsomes

of control and AKH-treated animals with or without the addition of exogenous MG (18), and found no effect of AKH on MGAT activity. The fact that MGAT activity measured in the presence of endogenous substrate showed no difference between AKH-treated and control insects suggested that the size of the microsomal MG pool was unaffected by AKH, and the data obtained in this study confirmed that suggestion. Given the results presented in this paper, which showed that the *sn*-2-MG pool size does not change during AKH-stimulated synthesis of *sn*-1,2-DG and the observation that AKH does not stimulate MGAT activity, it seems highly unlikely that the hydrolysis of TG to *sn*-2-MG followed by its reacylation is the pathway for the synthesis of *sn*-1,2-DG that are secreted by the insect fat body in response to AKH.

A second pathway by which DG could be formed is the *sn*-3-glycerophosphate route. Initially, fat body TG has to be hydrolyzed to glycerol and FFA. Glycerol would then be converted into glycerol-3-phosphate and esterified with FFA to produce PA, which after hydrolysis, produces *sn*-1,2-DG. The occurrence of this pathway for the synthesis of acylglycerols has been shown in insect fat body (35–37). In the case of *M. sexta*, it is known that injected radiolabeled FFA or glycerol is converted into TG, among other components, which are found in the fat body (38). Presumably, these TG are synthesized in the fat body by the *sn*-3-glycerophosphate pathway. If this pathway were important in the formation of DG stimulated by AKH, there should be an increase in the FFA and PA content of the fat body cell, after AKH treatment. However, the results presented here show that fatty acids from TG are converted to DG without increasing the fat body content of either FFA or PA. This fact suggests that the *sn*-3-glycerophosphate pathway does not play a role in the synthesis of DG stimulated by AKH, unless the reesterification of FFA occurs at the same rate as its formation, which seems unlikely.

The third alternative for the synthesis of *sn*-1,2-DG is the stereospecific hydrolysis of stored TG. The results of the precursor–product analysis reported here showed that the only significant change among the fat body radiolabeled lipid components induced by AKH was an accumulation of *sn*-1,2-DG, suggesting that stored TG is the direct precursor of secreted *sn*-1,2-DG. Therefore, it seems highly likely that the stereospecific hydrolysis of TG into DG is the pathway for the AKH-stimulated synthesis of *sn*-1,2-DG. This conclusion is also supported by the fact that activation of fat body TG-lipase activity by AKH precedes the appearance of DG in the hemolymph (20) and the absence of any effect of AKH on MGAT activity (18).

On the other hand, it has been shown that in vitro hydrolysis of triolein catalyzed by the purified fat body TG-lipase produces *sn*-2-MG instead of *sn*-1,2-DG (13). This discrepancy could be an artifact of the experimental conditions under which the TG-lipase activity was characterized in vitro, which involved the use of detergent micelles of TG as the substrate, whereas the in vivo substrate is a fat droplet, whose properties are unknown at this time. It is also possible that the synthesis of the DG to be exported is coupled by its secretion, in which case further hydrolysis of DG would be prevented by its rapid secretion into the hemolymph.

While it is clear that the glycerol backbone and the fatty acids at the *sn*-1 and -2 positions of fat body TG are directly converted to *sn*-1,2-DG, we have no information about the fate of the *sn*-3 fatty acid of the TG.

Conclusions

Based on the experiments reported here, it is concluded that the fatty acids at the *sn*-1 and -2 positions of fat body TG and the glycerol backbone of the TG are converted directly to *sn*-1,2-DG, without passing through any other intermediate. These results support the hypothesis that the AKH-stimulated synthesis of *sn*-1,2-DG in the fat body involves stereospecific hydrolysis of the TG stores.

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