In vitro study of the action of adipokinetic hormone in locusts

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Abstract The in vitro study was performed in order to demonstrate the structural changes of lipophorin induced in vivo by the injection of adipokinetic hormone (AKH) into adult locusts. After many unsuccessful attempts, we have established the reconstructed incubation system in which purified lipophorin and apolipophorin-III (9 mol/mol lipophorin) are incubated with the fat body in the presence of AKH under a supply of excess oxygen. In this system, high density lipophorin (HDL_p) originally present in the incubation medium can be transformed entirely into low density lipophorin (LDL_p) due to the loading of an increased amount of diacylglycerol from the fat body. The LDL_p formed in this incubation system was exactly the same as the LDL_p formed in vivo by the injection of AKH, in terms of density, particle size, diacylglycerol content, and the association with apolipophorin-III (apoLp-III). In the absence of apoLp-III, AKH did not exhibit its function to any extent. It was also demonstrated that the transformation of HDL_p to LDL_p requires calcium ions. Moreover, it appears that, up to a certain limit, the increase of diacylglycerol content of lipophorin and the amount of apoLp-III associated with lipophorin is nearly proportional to the amount of apoLp-III added to the incubation medium.—**Chino, H., Y. Kiyomoto, and K. Takahashi.** In vitro study of the action of adipokinetic hormone in locusts. *J. Lipid Res.* 1989. **30:** 571–578.

Supplementary key words lipophorin • apolipophorin-III • diacylglycerol • fat body • calcium ions

Lipophorin (1), a major hemolymph lipoprotein in insects, serves to transport, as a reusable shuttle, various lipids between tissues, and one of its functions is to deliver diacylglycerol as fuel from the fat body to the flight muscle (2, 3). In some insects such as the adult locust, the adult American cockroach (4), and the larval tobacco hornworm (5), lipophorin exists as high density lipophorin (HDL_p) and is composed of two apoproteins, apolipophorin-I (mol wt, approximately 250,000) and apolipophorin-II (mol wt, approximately 85,000).

Insect adipokinetic hormone (AKH), a blocked peptide (6) released from the corpora cardiaca, greatly stimulates the loading of lipophorin with diacylglycerol from the fat body and facilitates supply of diacylglycerol as fuel to the flight muscle during flight. Upon stimulation, lipophorin becomes considerably lower in density (low density lipophorin, LDL_p), larger, and heterogeneous in size due to the loading of an increased amount of diacylglycerol (7–10). The injection of AKH into adult insects also promotes the association of a third low molecular weight apoprotein, apolipophorin-III (apoLp-III, mol wt, 17,000–20,000) with the lipophorin particle (9–13); 9 mol of apoLp-III associate with each mole of lipophorin in locusts (13), while 16 mol of apoLp-III associate with each mole of lipophorin in tobacco hornworm, *Manduca sexta* (14). The locust apoLp-III is a glycosylated protein (containing fucose, mannose, and glucosamine) and exists in a free form in the hemolymph of resting adults (10, 13), whereas the *M. sexta* apoLp-III is a nonglycosylated protein and a small amount of apoLp-III always associates with the lipophorin particle (2 mol/mol lipophorin) even in the hemolymph of resting adults (12, 14, 15).

All the above changes observed for lipophorin upon injection of AKH are completely reversible; within 24 hr of the AKH injection, the apoLp-III dissociates from lipophorin, and the size and density of lipophorin return to the original values observed in resting adult insects (10). These reversible changes result from the comsumption of diacylglycerol as fuel at the flight muscle through the action of muscle lipase (16, 17).

Inasmuch as all the above changes in the structure of lipophorin have been observed mostly in vivo after the injection of AKH, it is necessary to demonstrate exactly these changes in vitro, in order to further elucidate the mechanism of AKH action and the role of apoLp-III. However, our previous attempts to demonstrate the above effects of AKH on the structure of lipophorin in vitro have been unsuccessful (10). Van Heusden et al. (17, 18) have demonstrated the production of LDL_p by incubating fat body with whole serum or with isolated lipophorin and apoLp-

Abbreviations: HDL_p, high density lipophorin; LDL_p, low density lipophorin; IDL_p, intermediate density lipophorin; apoLp-III, apolipophorin-III; AKH, insect adipokinetic hormone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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III of locusts. More recently, Van Der Horst et al. (19) have also reported the formation of hybrid LDL_p by incubating the mixture of locust lipophorin and locust apoLp-III or *M. sexta* apoLp-III with locust fat body in the presence of AKH and they have offered an interesting proposal concerning the possible role of apoLp-III. However, all these in vitro studies have only shown the partial formation of LDL_p; lipophorin originally present in the incubation medium was not entirely transformed into LDL_p but a significant amount of lipophorin remained as an intact HDL_p after incubation (17-19).

The locust lipophorin and apoLp-III purified from hemolymph of resting adults are now available in this laboratory (4, 13), and, therefore, it is now possible to test the reconstructed system in vitro, in which the entire population of lipophorin (HDL_p) can be transformed totally into LDL_p upon incubation with the fat body in the presence of AKH. We have recently established this system, which is reported in this paper with related experiments.

MATERIALS AND METHODS

Animals and collection of hemolymph

Adult locusts, *Locusta migratoria,* (3-5 weeks after final molt) were taken from colonies maintained in this laboratory. Hemolymph was collected from adult locusts using the "flushing method" by injecting EDTA-containing saline into the hemocoele (20).

Chemicals

Locust AKH, a blocked decapeptide, was purchased from Peninsula Laboratories (San Carlos, CA). Chromatographically pure monostearin obtained from Sigma (St. Louis, MO) was used as an internal standard when the amount of diacylglycerol was determined by Iatroscan (see below). All other chemicals were of analytical grade. Double-glass-redistilled water was used throughout.

Preparation of lipophorin and apolipophorin-III

High density lipophorin (HDL_p) was prepared from resting male and female adults (in some experiments, male adults only) according to a specific precipitation method (4), and dialyzed several times against large volumes of Ringer's solution (120 mM NaCl, 15 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM PIPES buffer, pH 7.0) before use. ApoLp-III was prepared according to the method developed in this laboratory (13). The locust apoLp-III consists of three molecular species which have different isoelectric points, and are therefore separable on DEAE-cellulose column chromatography (13). In the present experiment, however, the mixture of the three apoLp-IIIs obtained by gel filtration was used without further separation by DEAE-cellulose column chromatography. Instead, the fraction of apoLp-III separated by the first gel filtration on Ultragel AcA 44 column was applied again to a second gel filtration on the same column to eliminate the 17K protein which is a major contaminant (13). The purity of the final lyophilized preparation of apoLp-III was tested by a reverse-phase high-performance liquid chromatography (RP-HPLC, Japan Spectroscopic LC-800 system), which was run on a C_8 column (4.6 × 250 mm) with acetonitrile and water containing 1% trichloroacetic acid. The results indicated that the apoLp-III was still contaminated with a trace of the 17K protein and an unidentified protein (data not shown, but both were less than 2% of apoLp-III).

Incubation of hemolymph with fat body

In order to find the optimal incubation conditions, preliminary experiments were first carried out in which the hemolymph freshly collected from male adults was incubated with the fat body in the presence of AKH. Since, as will be described later, calcium ions are essential for the action of AKH, the freshly collected hemolymph was dialyzed several times against large volumes of Ringer's solution to completely remove the EDTA that had been added to prevent spontaneous aggregation of lipophorin during the hemolymph collection (21). The physiological conditions of the fat body possibly vary with each individual, and therefore, the fat body used for incubation was prepared as follows. The fat body was carefully dissected from male adults and washed twice with Ringer's solution. Since this organ is found on both sides of the digestive tract, the fat bodies from ten adults were divided longitudinally and pooled separately in two small beakers, so that each beaker contained tissue equivalent in amount to the fat bodies of five adults. Thus, only two incubations could be run at the same time; this allowed us to minimize the experimental errors. Each incubation medium contained 1 ml of dialyzed hemolymph (6-8 mg protein) and 50 μl of 2 μM AKH. The final concentration of AKH was 100 nM except when otherwise stated. The incubation beakers were placed into a moistened plastic cylinder (17 ×30 cm i.d.). Since the anatomy of the fat body indicates that this organ is associated with a highly developed tracheal system and our early observation also showed that the loading of diacylglycerol from fat body by lipophorin is an energy-dependent process (22), the plastic cylinder was filled with pure oxygen. The cylinder was constantly shaken in a water bath (32°C) during incubation. After incubating for 3 hr, the incubation medium was brought to 5 ml by addition of 150 mM NaCl, 50 mM phosphate buffer, pH 7.0, and then subjected to density gradient ultracentrifugation (see below).

Incubation of lipophorin and apolipophorin-III with fat body

The standard incubation medium contained 1.5-2.0 mg protein of purified lipophorin dissolved in 1 ml Ringer's

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solution and the appropriate amount of lyophilized apoLp-III. The amount of apoLp-III added was calculated according to the previous observation that 9 mol of apoLp-III associate with each mole of lipophorin in response to the injection of AKH (13).

Other incubation conditions were essentially the same as those described above for the incubation of hemolymph with the fat body. After incubating for 3 hr, the incubation medium was subjected to a density gradient ultracentrifugation as described above.

Density gradient ultracentrifugation

Lipophorin was isolated from the incubation medium by KBr density gradient ultracentrifugation essentially according to Shapiro, Keim, and Law (23) as described by Chino, Downer, and Takahashi (10). In order to compare the relative densities of the lipophorin fractions, a centrifugation time of 4 hr used in this method was sufficient. The density gradient ultracentrifugation was also carried out to isolate lipophorin from the hemolymph of AKH-injected locusts, as reference.

Determination of diacylglycerol

A certain amount of monostearin was first added to lipophorin fractions as an internal standard. The lipids were then extracted from the lipophorin fractions with chloroform–methanol 2:1 (v/v) and diacylglycerol was analyzed by Iatroscanner (model Th-10). This method is sensitive and highly reproducible, and is based on a combination of thin-layer chromatography (TLC) on coated quartz rods and flame ionization detection (24). The rods (Chromarod S-III), to which the samples were applied, were developed with benzene–methanol 40:1 (v/v) for 30 min. It was noted that the diacylglycerol determined by this method gave a value higher than that obtained by the TLC scanning method used in the previous study (10), particularly when a relatively large amount of lipid sample was applied to the rods. Although such a significant discrepancy exists between the two methods, we used the Iatroscanner in this study because of its high specificity and reliability.

Gel electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) of lipophorin was performed according to the method of Laemmli (25) and the gels were stained with Coomassie blue. The lipophorin fractions isolated by density gradient ultracentrifugation were dialyzed against 10 mM phosphate buffer (pH 6.9) to remove excess KBr before treatment with SDS.

Electron microscopy

The lipophorin preparations isolated by density gradient ultracentrifugation were first dialyzed against 150 mM KCl in 20 mM phosphate buffer (pH 6.0), and the samples (50 μg protein/ml) were applied to the supporting films, stained with 1% uranyl acetate, and observed in a Hitachi 11B electron microscope. The supporting films coated with carbon were made hydrophilic by ion bombardment before use.

Protein determination

Protein was determined by the Bio-Rad protein assay reagent (Bio-Rad Laboratories) and also the method of Lowry et al. (26) for comparison in some experiments, with bovine serum albumin as standard.

RESULTS

The stimulating effect of AKH on the loading of diacylglycerol from the fat body by lipophorin was confirmed by the injection of AKH (10 pmol/insect) into male adults, and the data were provided as references for the in vitro experiments. As reported in the previous paper (10), the density of lipophorin (LDL_p) isolated by density gradient ultracentrifugation from the AKH-injected hemolymph was 1.065 g/ml and considerably lower than the density of lipophorin (HDL_p, 1.12 g/ml) isolated from the hemolymph of resting locusts (data not shown). The amount of diacylglycerol in LDL_p was found to be 211 ± 13.1 μg/100 μg protein (mean ± SD) and about 7 times greater than that (30 ± 1.2 μg/100 μg protein) in HDL_p.

Incubation of hemolymph with fat body

Preliminary experiments were performed to determine the optimal incubation conditions under which the action of AKH is fully demonstrated in vitro. The freshly collected and dialyzed hemolymph was incubated with the fat body in the presence or absence of AKH under conditions described in Methods. After incubation, the lipophorin fraction was isolated from the incubation medium by density gradient ultracentrifugation. As illustrated in **Fig. 1**, the incubation of hemolymph with the fat body in the presence of 100 mM AKH resulted in a dramatic shift in the density of lipophorin from an original value of 1.12 g/ml (Fig. 1, a) to a lower value of 1.065 g/ml (Fig. 1, d) as observed in vivo after the injection of AKH. This clearly indicates that HDL_p originally present in the incubation medium is entirely transformed into LDL_p during incubation. The incubation at a lower concentration of AKH (20 nM) caused two distinct peaks corresponding to LDL_p and HDL_p but no lipophorin fraction having an intermediate density was observed (Fig. 1, c). When hemolymph was incubated with the fat body in the absence of AKH, the major lipophorin fraction appeared as HDL_p with a small peak of LDL_p (Fig. 1, b). In some experiments, however, the absence of AKH resulted in a rather broad band at a density of 1.08–1.09 g/ml in addition to the major HDL_p fraction. When hemolymph was incubated with fat body

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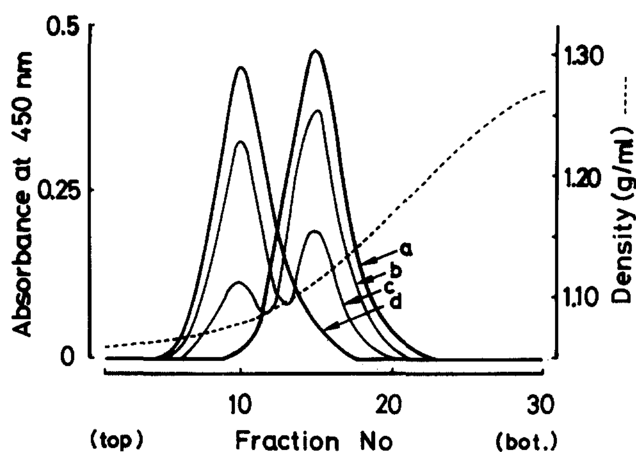

Fig. 1. Density gradient ultracentrifugation profiles of lipophorins after the incubation of hemolymph with the fat body in the presence or absence of AKH. The dialyzed hemolymph (6-7 mg protein) was incubated with the fat body under conditions described in Methods. After incubation, the medium was subjected to density gradient ultracentrifugation. After centrifugation, tubes were fractionated in 0.3-ml fractions from the top. The yellow band due to carotenoid pigments associated with lipophorin was monitored at 450 nm. a, Incubation without fat body (control); b, incubation in the absence of AKH; c, incubation in the presence of 20 nM AKH; d, incubation in the presence of 100 nM AKH.

in the presence of AKH under atmospheric conditions without an extra supply of oxygen, the major lipophorin fraction usually appeared as LDL_p but was always accompanied by a broad band at a density of 1.075-1.09 g/ml (data not shown). In this report, lipophorin having an intermediate density between LDL_p and HDL_p will be designated as intermediate density lipophorin (IDL_p).

Table 1 shows the changes of diacylglycerol content in lipophorin induced by the incubation of hemolymph in the presence of 100 nM AKH. The incubation resulted in a remarkable increase of diacylglycerol content in lipophorin, which was nearly equivalent to the increase observed in vivo after the injection of AKH.

The association of apoLp-III with lipophorin after the incubation of hemolymph with the fat body in the presence of AKH was examined by SDS-PAGE. The results (**Fig. 2**) clearly demonstrate that a considerable amount of apoLp-III associates with lipophorin after the incubation in the presence of AKH (lane 5) as well as after the injection of AKH (lane 1), whereas no detectable apoLp-III is associated with lipophorin (HDL_p) when incubated without the fat body (lane 4). The IDL_p formed in some experiments by the incubation in the absence of AKH contained only a small amount of apoLp-III (lane 6).

The electron micrograph of lipophorin (LDL_p) isolated by density gradient ultracentrifugation from the hemolymph incubated with the fat body in the presence of AKH is illustrated in **Fig. 3C** and demonstrates images similar to those of lipophorin isolated from the AKH-injected hemolymph (Fig. 3B); after incubation, the lipophorin particles become considerably larger and heterogeneous in size compared to HDL_p isolated from the hemolymph of resting locusts (compare A to B and C in Fig. 3).

Effect of calcium ions on the action of adipokinetic hormone

The possible effect of calcium ions on the action of AKH was tested in the in vitro system. The hemolymph freshly collected by the flushing method was first dialyzed several times against large volumes of Ca^{2+}-free Ringer's solution. The dialyzed hemolymph was then incubated with the fat body in the presence of AKH. The density gradient ultracentrifugation of the incubation medium demonstrated that the major fraction of lipophorin appeared as HDL_p with a faint broad band at d 1.10 g/ml. In contrast, all the lipophorin fractions were isolated as a single LDL_p peak when Ca^{2+} (4 mM) was previously added to the incubation medium (data not shown). Since it was found that the addition of EGTA (5 mM) to remove calcium possibly remaining in the fat body cells caused significant disintegration of the tissue, the use of this chelator was avoided in the present experiment. The effect of Ca^{2+} was not replaced by Mg^{2+} (2-5 mM).

The effect of Ca^{2+} on the changes in diacylglycerol content of lipophorin is shown in **Table 2**. The data demonstrate that the diacylglycerol content in lipophorin formed (IDL_p) by incubation in the absence of Ca^{2+} is rather low (only 1.6 times greater than the original value of HDL_p) compared to the diacylglycerol content of LDL_p formed by incubation in the presence of Ca^{2+} (about 6.5 times greater).

The effect of Ca^{2+} on the association of apoLp-III with lipophorin was also examined by SDS-PAGE. The results illustrated in Fig. 2 demonstrate that lipophorin (IDL_p) isolated from the medium incubated in the absence of Ca^{2+} associates with only a small amount of apoLp-III (lane 3).

TABLE 1. Changes in diacylglycerol content of lipophorin after the incubation of hemolymph with the fat body in the presence of AKH

Incubation Conditions	Lipophorin Class[a]	Diacylglycerol Amount	Diacylglycerol Ratio[b]
		µg/100 µg protein	
No fat body (control)	HDL_p	32 ± 2.9	1
AKH, 100 nM	LDL_p	201 ± 11.9	6.4 ± 0.39

Hemolymph (6-8 mg protein) was incubated under the conditions described in Methods. After incubation, lipophorin was isolated from the incubation medium by density gradient ultracentrifugation and the amount of diacylglycerol was determined. Values are expressed as mean ± SD of three or four experiments.

[a]Lipophorin class used for determination of the diacylglycerol content.
[b]Increase against control value.

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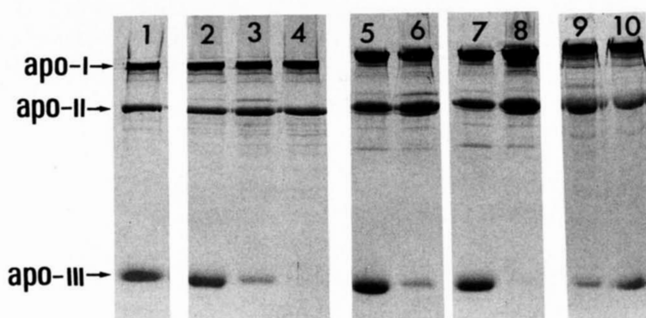

Fig. 2. SDS-polyacrylamide gel electrophoresis of lipophorins isolated by density gradient ultracentrifugation from the incubation media after incubation under various conditions. All the incubations contained fat body and 100 nM AKH except where stated. Lane 1, LDL_p isolated from the hemolymph of AKH-injected locusts (as a reference); lane 2, LDL_p from the incubation of Ca^{2+}-free hemolymph in the presence of 4 mM Ca^{2+}; lane 3, IDL_p (d 1.09–1.10 g/ml) from the incubation of Ca^{2+}-free hemolymph; lane 4, HDL_p from the incubation of hemolymph without fat body; lane 5, LDL_p from the incubation of hemolymph; lane 6, IDL_p (d 1.09 g/ml) from the incubation of hemolymph in the absence of AKH; lane 7, LDL_p from the incubation of purified lipophorin and 9 mol apoLp-III/mol lipophorin; lane 8, HDL_p from the incubation of lipophorin in the absence of apoLp-III; lane 9, IDL_p (d 1.075–1.08 g/ml) from the incubation of lipophorin in the presence of 5 mol apoLp-III; lane 10, LDL_p from the incubation of lipophorin in the presence of 9 mol apoLp-III.

In contrast, the extent of apoLp-III association with LDL_p formed in the presence of Ca^{2+} (lane 2) was nearly the same as that observed for LDL_p formed in vivo.

Amount of apolipophorin-III added affects the action of adipokinetic hormone

Fig. 4 illustrates the profiles of density gradient ultracentrifugation of lipophorins after purified lipophorin and different amounts of apoLp-III were incubated with the fat body in the presence of AKH. When no apoLp-III was added to the incubation medium, lipophorin was isolated as a single HDL_p peak at d 1.12 g/ml (Fig. 4a). In contrast, when 9 mol apoLp-III for each mole of lipophorin was added to the incubation medium, the lipophorin fraction appeared as a single peak of LDL_p at d 1.065 g/ml. When an intermediate amount of apoLp-III (e.g., 5.0 mol/mol lipophorin) was added to the incubation medium, the lipophorin was isolated as two peaks: one appeared as HDL_p and the other as IDL_p at d 1.075–1.08 g/ml (Fig. 4b). In some experiments, however, a peak corresponding exactly to LDL_p appeared in addition to the HDL_p peak after incubation in the presence of an intermediate amount of apoLp-III (data not shown).

The effect of apoLp-III on the diacylglycerol content of lipophorin was examined (**Table 3**). When lipophorin was incubated with the fat body in the absence of apoLp-III, there was only a slight increase of diacylglycerol content in the resulting lipophorin. On the other hand, when incubated in the presence of 9 mol apoLp-III/mol lipophorin, the resulting LDL_p contained a much increased amount of diacylglycerol, equivalent to the level of this lipid of LDL_p formed in vivo. The addition of an excess of apoLp-III (e.g., 12 mol/mol lipophorin) caused practically no further increase of diacylglycerol content. The IDL_p produced by the addition of 5 mol of apoLp-III to the incubation medium contained an increased amount of diacylglycerol but the increase was relatively low. The considerable association of apoLp-III with LDL_p produced by incubating lipophorin and 9 mol apoLp-III with the fat body in the presence of AKH was clearly demonstrated by SDS-PAGE (Fig. 2, lanes 7 and 10), whereas the SDS-PAGE pattern of IDL_p (d 1.08 g/ml) obtained after the incubation in the presence of 5 mol of apoLp-III showed a less dense band (lane 9).

The electron micrograph of lipophorin (LDL_p) isolated by density gradient ultracentrifugation after incubation in the presence of 9 mol of apoLp-III is illustrated in Fig. 3 D. The micrograph reveals clearly that the lipophorin particles become much larger and heterogeneous in size, as observed for lipophorin particles formed in vivo under the action of AKH (Fig. 3B).

All the experimental evidence presented here reveals that LDL_p produced by the incubation of lipophorin and 9 mol of apoLp-III with the fat body in the presence of AKH is exactly equivalent to LDL_p formed in vivo, in terms of density, diacylglycerol content, size, and the extent of apoLp-III association.

DISCUSSION

The major purpose of this study was to establish the incubation system in which the structural changes of lipophorin induced in vivo after the injection of AKH can be completely demonstrated in vitro. The preliminary experiments designed for this purpose provided satisfactory results; the incubation of freshly collected hemolymph with the fat body in the presence of AKH under the conditions described in the Methods section caused the entire transformation of HDL_p into LDL_p (Fig. 1). The incubation of hemolymph with the fat body also provided definite evidence that the existence of fat body is essential for the formation of LDL_p. The production of a small amount of LDL_p and/or IDL_p in addition to the major HDL_p fraction after the incubation of hemolymph with the fat body in the absence of AKH suggests that the fat body may already be exposed to AKH or other compounds, including neurotransmitters, which have an action similar to that of AKH (27) before the dissection of the fat body. This possibility, together with the fact that tissues such as the flight muscle where diacylglycerol is unloaded from lipophorin are lacking in the incubation system, may help explain the formation of LDL_p or IDL_p even in the absence of AKH. When incubated in the

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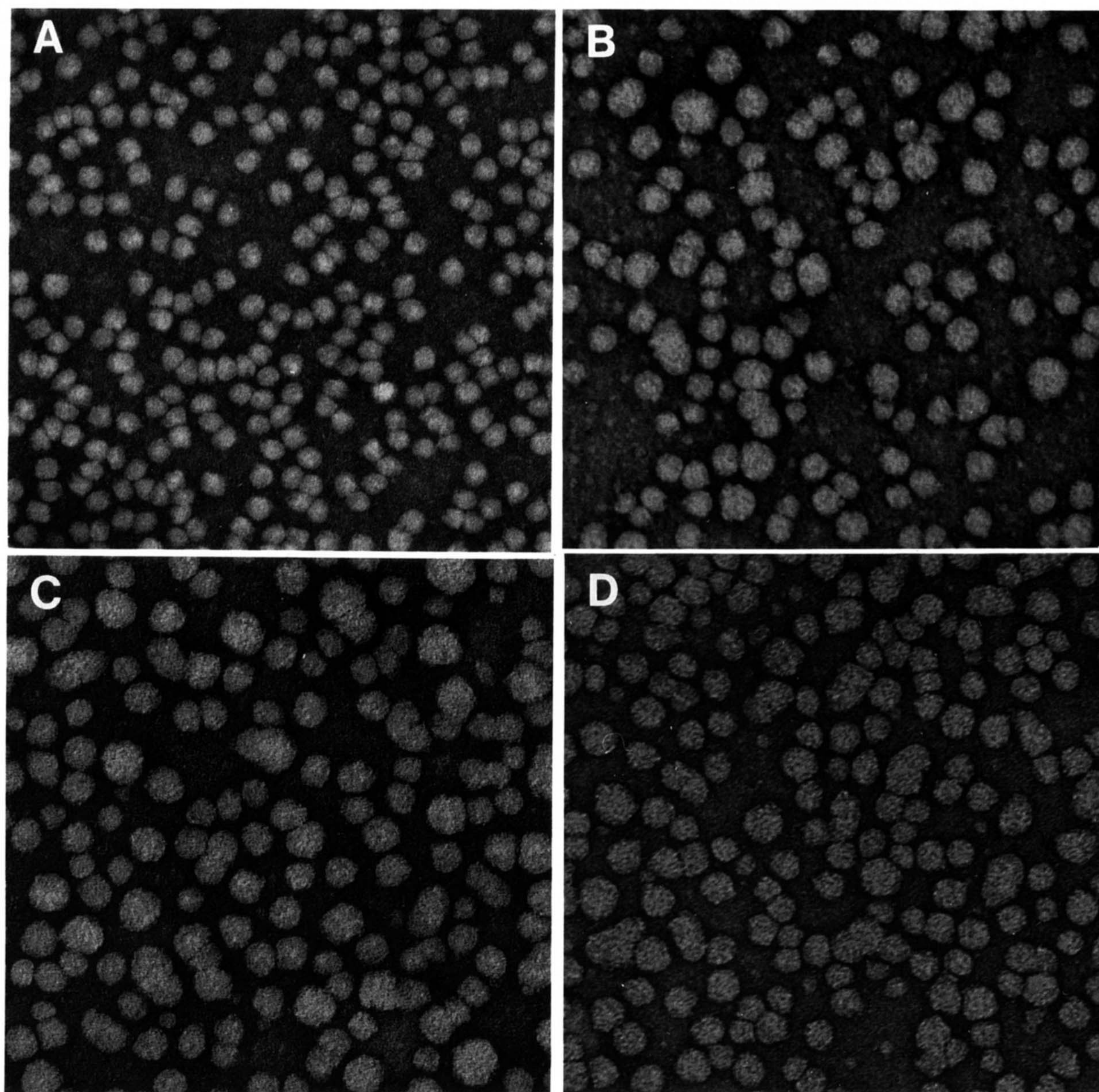

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Fig. 3. Electron micrographs of lipophorin particles. The lipophorins isolated by density gradient ultracentrifugation from different samples were negatively stained with uranyl acetate (× 200,000). A, lipophorin (HDL_p) from the hemolymph of resting locusts; B, lipophorin (LDL_p) from the hemolymph of AKH-injected locusts; C, lipophorin (LDL_p) from the incubation of hemolymph with the fat body in the presence of AKH; D, lipophorin (LDL_p) from the incubation of purified lipophorin and 9 mol apoLp-III/mol lipophorin with the fat body in the presence of AKH.

presence of AKH at a lower concentration (20 nM), the density gradient ultracentrifugation of the incubation medium results in two distinct peaks corresponding to LDL_p and HDL_p, respectively, but no peak is observed at an intermediate density (Fig. 1 c). This result coincides with our previous observation that when an insufficient amount of AKH is injected into locusts, two peaks of LDL_p and HDL_p are found but no peak appears at an intermediate density (10). These results imply that the response of lipophorin to AKH action is all or nothing.

Spencer and Candy (28) have reported that calcium ions are required for optimum mobilization of diacylglycerol from the fat body into the hemolymph in the desert locust. The effect of calcium ions on the formation of LDL_p in the present study is remarkable; the omission of Ca^{2+} from the incubation medium completely abolished the formation of LDL_p, although a small amount of IDL_p (d 1.10–1.11 g/ml) is produced even in the absence of Ca^{2+}. The production of IDL_p is very possibly due to the presence of traces of Ca^{2+} remaining in the fat body cells. These observations

TABLE 2. Effect of Ca^{2+} on the diacylglycerol content of lipophorin during the incubation of Ca^{2+}-free hemolymph with the fat body in the presence of 100 nM AKH

Incubation Conditions	Lipophorin Class	Diacylglycerol	
		Amount	Ratio
		μg/100 μg protein	
No fat body (control)	HDL_p	31 ± 1.6	1
No Ca^{2+}	IDL_p[a]	49 ± 2.2	1.6 ± 0.09
Ca^{2+}, 4 mM	LDL_p	200 ± 16.8	6.5 ± 0.45

The freshly collected hemolymph was dialyzed several times against large volumes of Ca^{2+}-free Ringer solution before incubation.
[a]Density = 1.10 g/ml.

TABLE 3. Effect of apoLp-III on the diacylglycerol content of lipophorin during the incubation of purified lipophorin with the fat body in the presence of 100 nM AKH

Incubation Conditions	Lipophorin Class	Diacylglycerol	
		Amount	Ratio
		μg/100 μg protein	
No fat body (control)	HDL_p	30 ± 2.1	1
No apoLp-III	HDL_p	37 ± 1.9	1.2 ± 0.05
ApoLp-III, 5 mol	IDL_p[a]	119 ± 17.0	4.0 ± 0.22
ApoLp-III, 9 mol	LDL_p	197 ± 6.0	6.6 ± 0.25
ApoLp-III, 12 mol	LDL_p	206 ± 9.5	6.8 ± 0.31

Purified lipophorin (1.5-2.0 mg protein) was incubated under the conditions described in Methods. The amount of apoLp-III added is expressed as moles/each mole lipophorin.
[a]Density = 1.075-1.08 g/ml.

concerning the effect of Ca^{2+} seem important to the investigation of the action site of AKH. It is possible that AKH primarily stimulates, via a Ca^{2+}-dependent protein kinase, a fat body lipase, which causes the level of diacylglycerol in the fat body cells to increase, and consequently results in the loading of an increased amount of this lipid by lipophorin. We are now testing this possibility.

The most significant result in the present study is the establishment of the reconstructed incubation system to demonstrate in vitro the structural changes in lipophorin observed in vivo after the injection of AKH; the incubation of purified lipophorin and apoLp-III (9 mol/mol lipophorin) with the fat body in the presence of AKH under the conditions described in Methods allows the transformation of the entire HDL_p population originally present in the incubation medium into LDL_p. The establishment of this incubation system permits further in vitro investigation of the mechanism of AKH action including the role of apoLp-III. A recent paper from another laboratory (19) reported the production of LDL_p when locust lipophorin and apoLp-III are incubated with the locust fat body in the presence of AKH. Calculating on the basis of molecular weight, they added about 16 mol of apoLp-III/mol lipophorin to their incubation medium. In their results, however, the HDL_p fraction was not entirely transformed into LDL_p but a fraction of HDL_p always accompanied the LDL_p fraction. The partial formation of LDL_p from HDL_p may partly be due to the insufficient amount of fat body, compared to the amount of lipophorin, in their incubation system.

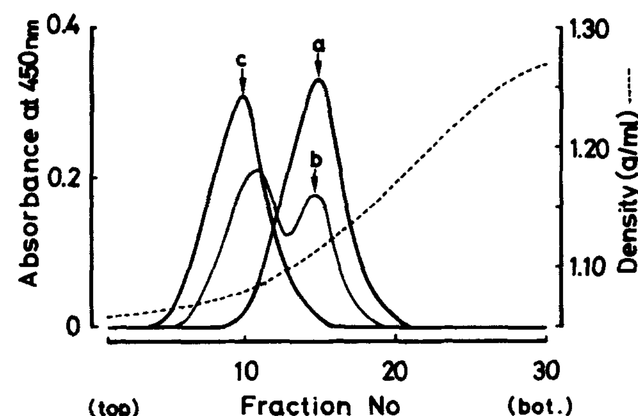

Fig. 4. Effect of apoLp-III on the density of lipophorin. The purified lipophorin (1.5-2.0 mg protein) and various amounts of apoLp-III were incubated with the fat body in the presence of 100 nM AKH (see Methods for details). After incubation, the incubation medium was subjected to density gradient ultracentrifugation. See also legend to Fig. 1. a, Incubation in the absence of apoLp-III; b, incubation in the presence of 5 mol apoLp-III/mol lipophorin; d, incubation in the presence of 9 mol apoLp-III.

The omission of apoLp-III from the incubation medium caused no production of either LDL_p or even IDL_p, although the diacylglycerol content in lipophorin increased slightly after the incubation (Table 3). This slight increase of diacylglycerol content seems only natural, because the lipophorin itself has a capacity to accept, to a small extent, diacylglycerol from the fat body (2, 4). The addition of intermediate amounts of apoLp-III (e.g., 5 mol/mol lipophorin) usually results in the production of IDL_p (d 1.075-1.08 g/ml) with an increased amount of diacylglycerol to an intermediate level and a less dense band of apoLp-III on the SDS-PAGE pattern (Table 3 and Fig. 2). These data appear to indicate that, up to a certain limit, the amount of diacylglycerol loaded by lipophorin from the fat body and also the amount of apoLp-III associated with lipophorin are nearly proportional to the amount of apoLp-III added in the incubation medium. As mentioned earlier in the Results section, an LDL_p fraction and not an IDL_p fraction was sometimes isolated from the incubation medium when incubated in the presence of 5 mol apoLp-III. At present, it is difficult to define the incubation conditions under which LDL_p is produced even in the presence of such an insufficient amount of apoLp-III.

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The present result showing that the addition of 9 mol apoLp-III/mol lipophorin to the incubation medium causes the complete transformation of HDL_p to LDL_p appears to confirm our previous proposal that the maximum number of molecules of apoLp-III associated with LDL_p formed in vivo are 9 mol for each mol lipophorin. However, other investigators (19) have recently reported that hybrid LDL_p formed by incubating locust lipophorin and *M. sexta* apoLp-III with the locust fat body in the presence of AKH contained 14 mol of apoLp-III/mol lipophorin. The problem of how many molecules of apoLp-III associate with lipophorin under different conditions remains an important question, which must be resolved using more reliable techniques.

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