Effect of adipokinetic hormone on the structure and properties of lipophorin in locusts

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Abstract The reversible association of a low molecular weight hemolymph protein (mol wt 20,000 estimated by SDS-polyacrylamide gel electrophoresis) with lipophorin, following treatment with adipokinetic hormone (AKH), was demonstrated by density gradient ultracentrifugation and by specific precipitation of lipophorin from the hemolymph of resting and AKH-injected locusts. The injection of AKH also stimulated the loading of diacylglycerol from fat body by lipophorin and resulted in a lower density lipophorin ("activated lipophorin"). The activated lipophorin particles (diameter 21.7 ± 3.0 nm, 15.8 to 33.6 nm) were larger and more heterogeneous in size than those of resting lipophorin (14.5 ± 1.6 nm, 11.9 to 19.2 nm). A theoretical analysis based on the experimental data (e.g., density gradient profile, electron microscopic observation, and diacylglycerol content) suggests that very large lipophorin particles result from intermolecular fusion of the lipophorin molecules that are activated by AKH. Attempts to demonstrate the effect of AKH on the structure of lipophorin, in vitro, were unsuccessful. —**Chino, H., R. G. H. Downer, and K. Takahashi.** Effect of adipokinetic hormone on the structure and properties of lipophorin in locusts. *J. Lipid Res.* 1986. **27:** 21–29.

Supplementary key words diacylglycerol • apolipophorin III • density gradient ultracentrifuge • electron microscopy

The hemolymph lipoprotein profile of adult locusts has been studied in resting insects and immediately following flight (1–3). In resting insects, diacylglycerol, the major lipid component of insect hemolymph (4), is associated principally with a single lipoprotein; however, following flight or after the injection of an aqueous extract of corpora cardiaca or purified adipokinetic hormone (AKH) most of the diacylglycerol appears to be bound to a different higher molecular weight lipoprotein (1–3). The appearance of the second lipoprotein in hemolymph occurs concomitantly with a decrease in the concentration of a low molecular weight, non-lipid-containing protein (5). These observations suggest that the high molecular weight lipoprotein, which appears during flight or after injection of AKH, results from association of the low molecular weight hemolymph protein and the major diacylglycerol-containing lipoprotein of resting insects (6–8).

The high diacylglycerol content of the lipoprotein from resting hemolymph, together with the distinctive yellow coloration, indicate that this lipoprotein is lipophorin, the major vehicle for lipid transport in insects (9). In the tobacco hornworm, *Manduca sexta,* lipophorin comprises two apoproteins with molecular weights of 245,000 and 78,000, respectively (10). Injection of AKH into adult *M. sexta* results in the reversible association of a third apoprotein (mol wt, 17,000) with lipophorin to form a lower density lipophorin particle (11, 12). We have reported previously (13) that the subunit structure of lipophorin from the hemolymph of resting locusts also comprises two apoproteins with molecular weights of 250,000 and 85,000, respectively, and that insect lipophorins display no species-specificity in their function. It was, therefore, of interest to determine whether the flight- and hormone-induced, high molecular weight lipophorin reported for locusts (1–3) is equivalent to the low density lipophorin particle of *M. sexta.*

The present study describes the purification and partial characterization of lipophorin particles from the hemolymph of resting and AKH-injected adult locusts. The lipophorin particle purified from AKH-treated locusts demonstrates the presence of a third apoprotein. The AKH-induced lipophorin also demonstrates an increase in size and decrease in density due to the loading of additional diacylglycerol. Thus the results are consistent with the proposal that the lipophorin complex exists in "resting" and "activated" forms in the hemolymph of, at least, some Orthoptera and Lepidoptera. It is also suggested that very large lipophorin particles, which have been observed (2), may result from intermolecular fusion of AKH-activated lipophorin molecules.

Abbreviations: Apo-III, apolipophorin-III; AKH, insect adipokinetic hormone; SDS, sodium dodecyl sulfate.

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MATERIALS AND METHODS

Animals and collection of hemolymph

Adult locusts, *Locusta migratoria,* (3–5 weeks after the final molt) were taken from colonies maintained in this laboratory. Hemolymph was collected from resting and AKH-injected locusts by a "flushing out" method that is described in detail in a previous paper (14).

Chemicals

Adipokinetic hormone (15), a blocked decapeptide (pyroGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH$_2$), was purchased from Peninsula Laboratories. Standard proteins of known molecular weight were obtained from Boehringer Mannheim. Florisil (60–100 mesh) and DEAE-cellulose were obtained from Sigma Chemical Co., and [1-^{14}C]palmitic acid (50 mCi/mmol) was from New England Nuclear Corp. Chromatographically pure diolein was prepared in this laboratory (16). All other chemicals were of analytical grade and solvents were redistilled as appropriate. Glass-redistilled water was used throughout the experiments.

Isolation of lipophorin from locust hemolymph

Density gradient ultracentrifugation. Lipophorin was isolated from freshly collected locust hemolymph by potassium bromide density gradient centrifugation using a procedure similar to that employed by Shapiro, Keim, and Law (10) for *M. sexta.* Hemolymph was collected from resting and AKH-injected male locusts (usually 13–15 animals for each run) by flushing the hemocoel with about 0.35 ml of saline (150 mM NaCl, 5 mM EDTA in 50 mM phosphate buffer, pH 6.8). The pooled hemolymph (approximately 5 ml) was then centrifuged at 2,000 *g* (5°C) for 5 min to remove the hemocytes and 2.2 g of KBr was added, with stirring, to 5 ml of the supernatant to give a final density of 1.31 g/ml. The KBr–hemolymph mixture (5.5 ml) was placed in a 12-ml centrifuge tube and overlayered with 5.5 ml of 0.9% NaCl (density=1.007). The tube was placed in a Hitachi 65-P vertical rotor and centrifuged at 50,000 rpm for 4 hr at 4°C in a Hitachi ultracentrifuge (model 55P-7). After centrifugation, the sample was fractionated by a density gradient fractionator (ISCO, model 640). A tube lacking hemolymph was centrifuged and fractionated in the same way and the density gradient was determined by measuring the conductivity of each fraction in a conductivity meter (M & S Instruments Inc., model CD-35MII) and comparing the conductivity with that obtained for KBr solution of known density. Under these conditions, the density gradient ranged from 1.03 to 1.30 g/ml (**Fig. 1**). Lipophorin appeared as a single distinctive yellow band in the center (resting lipophorin Fig. 1A) and upper third (activated lipophorin Fig. 1B) of the tube. A biliverdin-binding cyano-protein (17) appeared as a broad blue band in the lower third of the tube. In some experiments, the darkest segment (about 0.7 ml) of the yellow band was collected directly by a Pasteur pipet and used as a source of resting or activated lipophorin for further analyses.

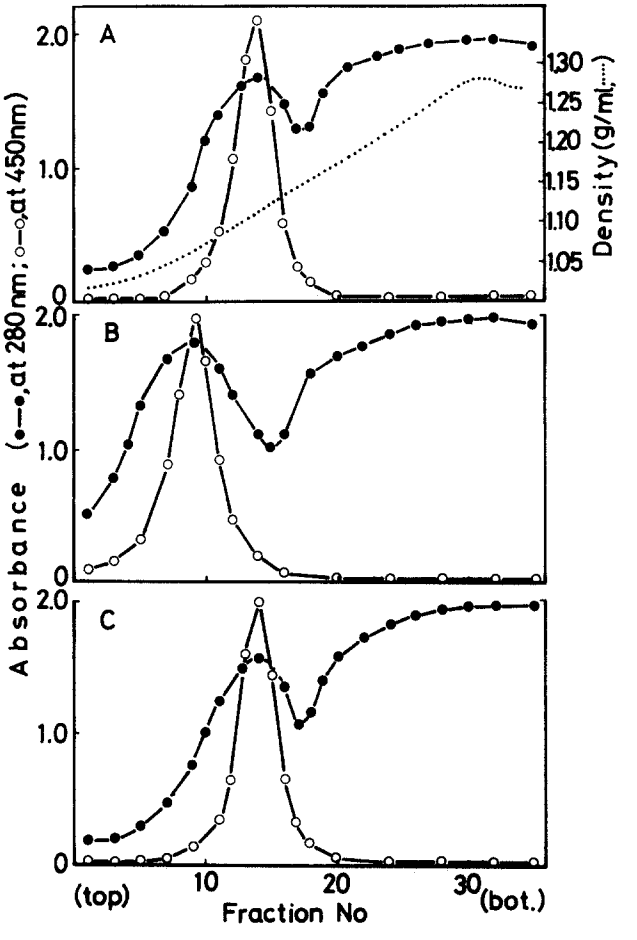

Fig. 1. Effect of AKH-treatment on the density of lipophorin particles from locust hemolymph. A, 90 min following injection of saline (resting lipophorin); B, 90 min following injection of AKH (activated lipophorin); C, 24 hr following injection of AKH. Thirteen adult male locusts were injected with AKH [10 pmol in phosphate saline (150 mM potassium chloride in 50 mM phosphate buffer, pH 6.8)] or saline and held at 30°C until hemolymph collection. Following density gradient ultracentrifugation of the hemolymph sample, tubes were fractionated in 0.3-ml fractions from the top. The yellow band was monitored at 450 nm.

Specific precipitation method. As indicated in previous publications (13, 18), the precipitation of lipophorin is facilitated by the presence of vitellogenin (female-specific protein) which acts as a co-precipitant of lipophorin. Therefore, female locusts were used as a source of the hemolymph from which lipophorin was purified by this method. Hemolymph was collected from resting and AKH-treated locusts by flushing out the hemocoel with saline (150 mM KCl, 5 mM EDTA in 20 mM phosphate buffer, pH 6.0). The pooled hemolymph was centrifuged at 2,000 *g* for 5 min at 5°C and the resulting hemocyte-free hemolymph was subjected to the previously described procedure (13).

Lipid extraction, separation, and determination

Neutral lipids were extracted from hemolymph, lipophorin, or incubation media by the procedure of Dole (19), and subjected to further separation on a Florisil column (20) or by thin-layer chromatography. The diacylglycerol content of lipophorin was determined by thin-layer chromatography using the following procedure. The neutral lipid fraction and several known amounts of diacylglycerol (diolein) were first applied to a plate (silica gel, Merck) which was developed by a solvent system comprising hexane–ethyl ether 25:75. The plate was then sprayed with saturated $K_2Cr_2O_7$ in 70% sulfuric acid and charred. The spots of diacylglycerol were scanned in a Shimadzu chromatographic scanner (model, CS-900).

Gel electrophoresis

Native polyacrylamide gel electrophoresis was performed in a 3.75% gel and the gel was stained with Coomassie blue G-250 by a rapid staining method (21). When lipophorin was prepared by the density gradient method, the sample was first dialyzed against 150 mM KCl in 20 mM phosphate buffer (pH 6.0) for 3 hr to remove excess KBr. SDS-polyacrylamide gel electrophoresis was performed after the method of Weber and Osborn (22) and the gel was stained with Coomassie blue. Lipophorin prepared by the density gradient method was dialyzed against 10 mM phosphate buffer (pH 6.9) for 4 hr before treatment of SDS.

Electron microscopy

The supporting films coated with carbon were made hydrophilic by ion bombardment before use. The lipophorin preparations were first dialyzed against 150 mM KCl in 20 mM phosphate buffer, pH 6.0; the samples (80 μg/ml) were applied to the supporting films, stained with 1% uranyl acetate, and observed in a Hitachi 11B electron microscope.

Protein determination

The amount of protein was determined by the Bio-Rad protein assay reagent (Bio-Rad Laboratories) using bovine serum albumin as standard.

RESULTS

Reversible change of density and size of lipophorin induced by adipokinetic hormone

The effect of AKH-treatment (10 pmol/insect) on the density of lipophorin particles isolated from locust hemolymph by density gradient centrifugation is illustrated in Fig. 1. The results indicate that treatment with AKH causes a change in the density of lipophorin particles from a resting value of 1.12 g/ml (Fig. 1A) to an activated value of 1.065 g/ml (Fig. 1B). The density of the lipophorin particle returns to the resting value within 24 hr of AKH treatment (Fig. 1C). Injection of a lower dose of AKH (3 pmol or less/insect) resulted in some low-density and some high-density lipophorin, but no peak of intermediate density was apparent (data not shown).

The diacylglycerol content values of lipophorin prepared from resting and AKH-injected locusts by density gradient ultracentrifugation are given in **Table 1.** It is evident that, 90 min following AKH injection, the diacylglycerol component of lipophorin increases by almost three times (based on protein content) over that of resting lipophorin and, within 24 hr of AKH injection, the diacylglycerol content returns to the original level. Other lipid components including free cholesterol and hydrocarbons were not affected by AKH injection (data not shown). These results suggest that the injection of AKH stimulates the loading of diacylglycerol from fat body by lipophorin and that the increased lipid content of the lipophorin complex results in the lower density lipophorin.

The specific precipitation method that has been developed in this laboratory (13) to purify lipophorin from resting locusts was tested to determine whether the method could be applied to the purification of activated lipophorin. The results confirmed that the method could be used for this purpose, although the recovery (66.4%) of activated lipophorin was slightly lower than that (72.7%) of resting lipophorin.

The size of lipophorin particles, purified by density gradient centrifugation of hemolymph from resting and AKH-treated locusts, was examined by electron microscopy of negatively stained particles. Electron micrographs of lipophorin particles from resting and AKH-injected locusts are illustrated in **Fig. 2** and measurements of particle diameter are presented in **Fig. 3.** The results indicated homogeneity in the diameter of resting lipophorin particles (Fig. 2A) with a mean diameter of 14.5

TABLE 1. *Diacylglycerol and protein content of lipophorin from resting and AKH-injected locusts*

Treatment	Content[a] Protein (A)	Diacylglycerol (B)	A : B
	mg	*mg*	
Resting	4.37	1.18	1 : 0.27
	5.10	1.21	1 : 0.24
AKH-injected (90 min)	4.62	3.35	1 : 0.73
	4.88	3.61	1 : 0.74
AKH-injected (24 hr)	4.75	1.33	1 : 0.28
	4.51	1.17	1 : 0.26

[a] Five fractions (total volume 1.5 ml) centered around the peak of absorbance at 450 nm (Fig. 1) werre combined and analyzed for protein and diacylglycerol content. The results from two experiments are presented for each treatment.

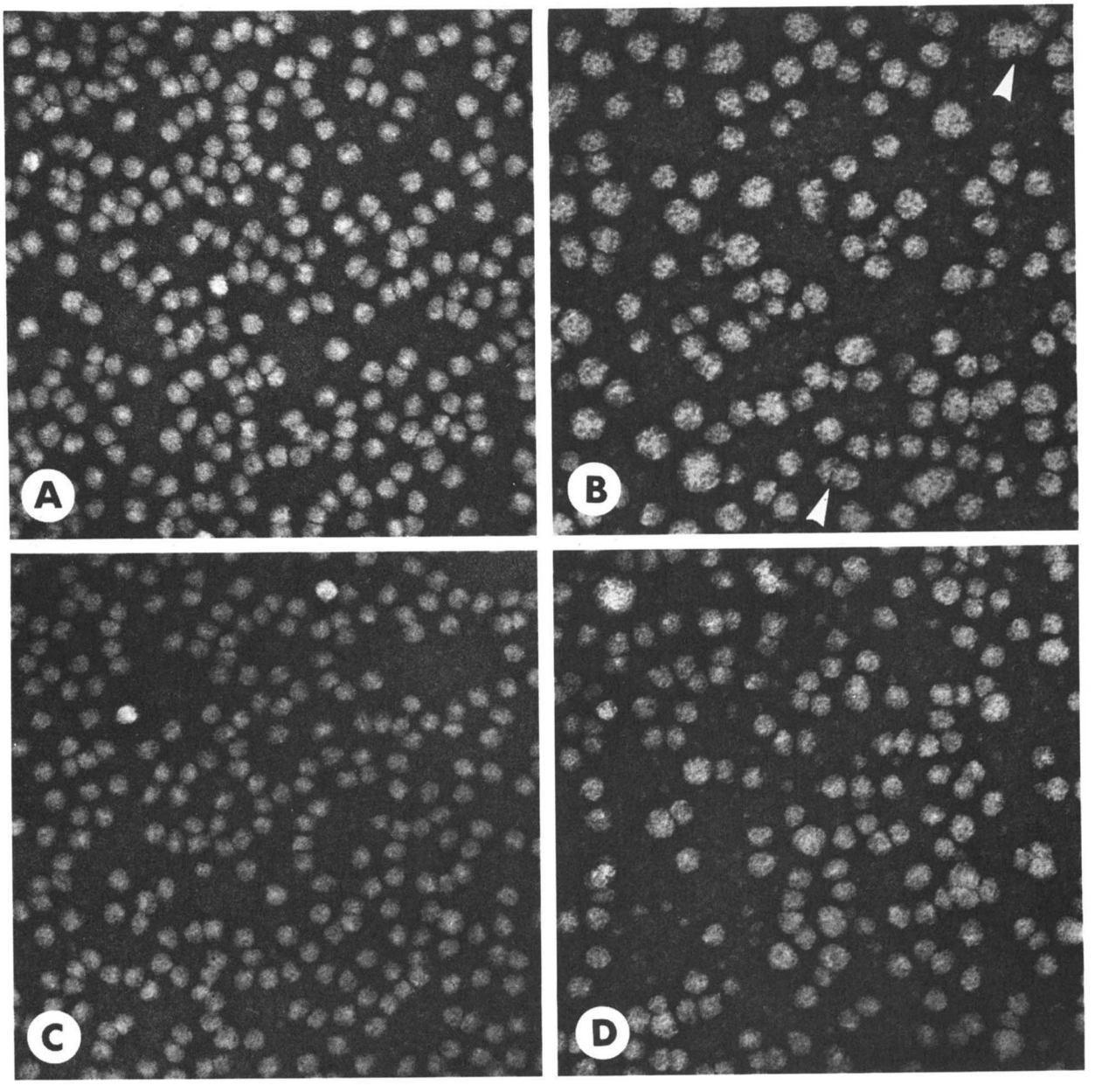

Fig. 2. Electron micrographs of resting and activated lipophorins negatively stained with uranyl acetate (× 200,000). A, 90 min following injection of saline (resting lipophorin); B, 90 min following injection of AKH (activated lipophorin); C, 24 hr following injection of AKH; D, 90 min following injection of AKH (activated lipophorin). A, B, and C were prepared by density gradient centrifugation whereas D was prepared by precipitation.

± 1.6 nm (Fig. 3A). Treatment with AKH resulted in greater heterogeneity (Fig. 2B) and, within 90 min of injection, the mean diameter of the lipophorin particles increased to 21.7 ± 3.0 nm (Fig. 3B). However, within 24 hr following the injection of AKH, the lipophorin particles returned to the smaller size and were homogeneous (Figs. 2C, 3C). Activated lipophorin particles prepared by the precipitation method resembled those derived by density gradient centrifugation (Fig. 2D), although the proportion of smaller size particles was greater when the precipitation procedure was employed (mean diameter 18.0 ± 2.8 nm; range 12.9–29.5 nm).

Examination of resting and activated lipophorin by native polyacrylamide gel electrophoresis supported the observations with electron microscopy. Resting lipophorin migrated as a distinct single sharp band on the gel, whereas activated lipophorin appeared as a broad diffuse band and migrated more slowly than resting lipophorin (**Fig. 4**).

Reversible association of apolipophorin-III with lipophorin under the action of adipokinetic hormone

SDS-polyacrylamide gel electrophoresis was used to

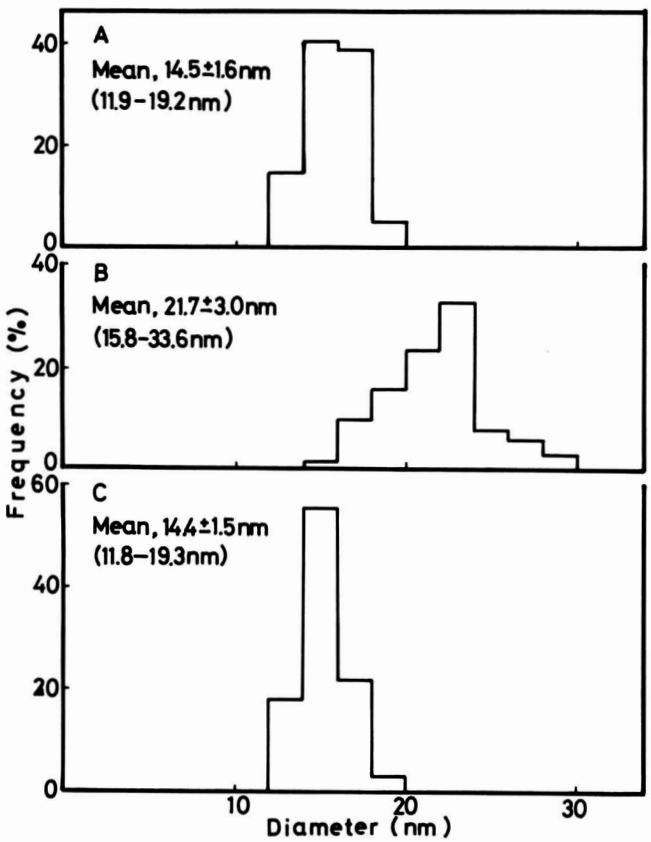

Fig. 3. Diameter of resting and activated lipophorin particles. Values indicate mean ± standard error of 480 particles within a specific region of the electron micrograph at a magnification of × 200,000. A, 90 min following injection of saline (resting lipophorin); B, 90 min following injection of AKH (activated lipophorin); C, 24 hr following injection of AKH.

demonstrate that AKH-injection causes the association of low molecular weight apoprotein with lipophorin. The results are illustrated in **Fig. 5** and demonstrate that a low molecular weight apoprotein appears in activated lipophorin prepared by density gradient centrifugation or by specific precipitation, whereas no such apoprotein is detected in resting lipophorin or lipophorin prepared 24 hr after the injection of AKH. This indicates that the association of low molecular weight apoprotein with lipophorin is a reversible process. In this report, the low molecular weight apoprotein is designated as apolipophorin III (Apo-III) following the terminology proposed by Shapiro and Law (11) for *M. sexta*. The molecular weight of Apo-III was determined by running the activated lipophorin, together with several standard proteins, on SDS-polyacrylamide gel electrophoresis. The molecular weight was estimated to be approximately 20,000 (**Fig. 6**).

Free Apo-III in hemolymph and its response to adipokinetic hormone

The above observations, together with several reports for *L. migratoria* (6–8) and *M. sexta* (11, 12), suggest that a low molecular weight hemolymph-protein (Apo-III) associates reversibly with lipophorin following the injection of AKH. Further evidence in support of this conclusion was obtained by subjecting freshly collected hemolymph and the lipophorin-free subnatant fraction obtained after density gradient ultracentrifugation to analysis by SDS-polyacrylamide gel electrophoresis. The electropherograms demonstrated that the concentration of Apo-III in hemolymph is not affected appreciably by AKH-injection (**Fig. 7a, b**). By contrast, the concentration of free Apo-III recovered in the subnatant fraction considerably decreased in response to AKH-treatment, whereas the concentrations of other hemolymph proteins recovered in the subnatant fraction did not change (Fig. 7c, d). This indicates that no additional Apo-III is newly released into hemolymph in response to AKH; rather the free Apo-III already present in hemolymph becomes associated with lipophorin under the influence of AKH.

Effect of adipokinetic hormone on lipophorin in vitro

In order to test whether AKH acts directly on the lipophorin complex to promote association with Apo-III, fresh hemolymph collected by the flushing-out procedure was incubated at 25–27°C in the presence or absence of AKH. After incubation, the incubation mixtures were subjected to density gradient ultracentrifugation and the lipophorin fraction was then tested for the association of

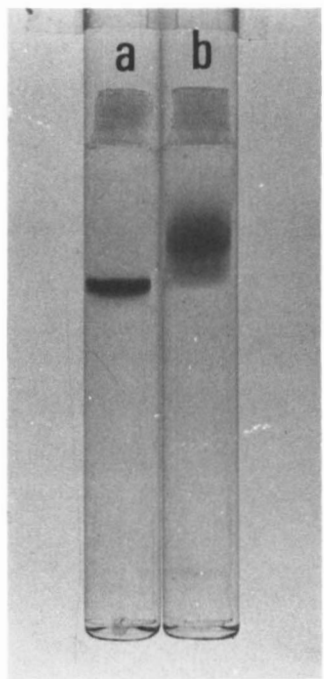

Fig. 4. Native polyacrylamide gel electrophoresis of resting and activated lipophorin particles purified by density gradient centrifugation. a, Resting lipophorin (15 μg of protein); b, activated lipophorin (15 μg of protein) from locusts 90 min after injection of 10 pmol of AKH.

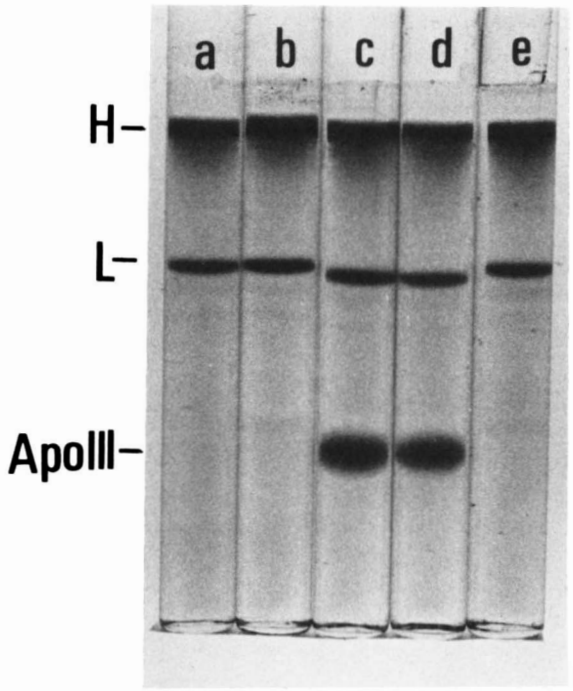

Fig. 5. SDS-polyacrylamide gel electrophoresis of resting and activated lipophorins [15 μg of lipophorin applied to each gel (5% gel)]. a, Lipophorin prepared from resting male locusts by density gradient ultracentrifugation; b, lipophorin prepared from resting female locusts by precipitation procedure; c, lipophorin prepared from AKH-injected male locusts by density gradient centrifugation 90 min following injection of 10 pmol of AKH; d, lipophorin prepared from AKH-injected female locusts by precipitation procedure 90 min following the injection of 20 pmol of AKH; e, lipophorin prepared from AKH- injected male locusts by density gradient centrifugation 24 hr following the injection of 10 pmol of AKH. H, heavy chain; L, light chain; Apo-III, apolipophorin III.

Apo-III by SDS-polyacrylamide gel electrophoresis. A variety of incubation conditions was employed including a range of hemolymph concentrations (1–5 mg/ml), a range of AKH concentrations (100–500 nM), different buffers (phosphate, Tris–HCl, PIPES; pH 6.0–7.2), different incubation times (60 or 90 min) and with or without calcium or magnesium. However, no appreciable association of Apo-III with lipophorin was detected. Attempts to use more concentrated or undiluted hemolymph for incubation were unsuccessful because such preparations rapidly became turbid and clotted.

DISCUSSION

Several authors (1–3, 6–8) have reported that the injection of AKH, or prolonged flight, lead to the appearance in locust hemolymph of a high molecular weight protein (fraction A^+) which is associated with the increased amounts of diacylglycerol observed in hemolymph under these conditions. The appearance of fraction A^+ occurs concomitantly with a decrease in the concentration of a non-lipid-containing protein (fraction C) and it has been suggested that A^+ may result from the association of fraction C with another lipoprotein (fraction A) which is present in the hemolymph of resting locusts. In the present account, the terms "activated lipophorin" and "resting lipophorin" are used to describe lipoprotein complexes that appear to be equivalent to fraction A^+ and fraction A, respectively, and apolipophorin-III (Apo-III) is used to describe a hemolymph protein that is equivalent to fraction C. The terminology used in this account is consistent with proposals that have been described in the literature (9, 12). The results of the present study support the proposal (1–3, 6–8) that injection of AKH into the locust hemocoel causes a low molecular weight protein in hemolymph to associate reversibly with the major diacylglycerol-carrying lipoprotein, lipophorin. AKH-injection also stimulates the loading of diacylglycerol from the fat body by lipophorin and results in a larger, lower density lipophorin particle similar to that which has been described following AKH-injection in *Manduca sexta*.

The data do not, however, indicate the amount of diacylglycerol that is loaded onto each lipophorin particle. Electron micrographs of the activated lipophorin demonstrate considerable heterogeneity in particle size, whereas those of the resting lipophorin display homogeneity in size (Figs. 2, 3). The heterogeneity of activated lipophorin

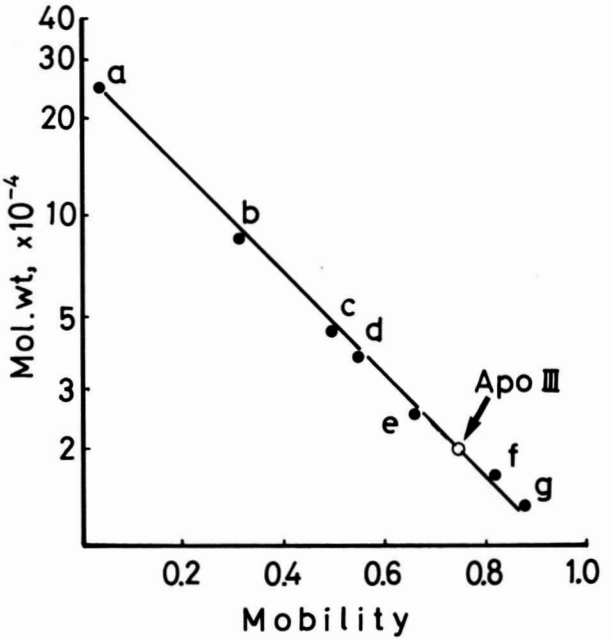

Fig. 6. Relationship between the molecular weights of standard proteins and apolipophorin-III and their mobilities on SDS-polyacrylamide gel electrophoresis. Runs were performed on 7.5% gels. a, Lipophorin heavy chain (250,000); b, lipophorin light chain (85,000); c, egg albumin (45,000); d, alcohol dehydrogenase (37,000); e, chymotrypsinogen (25,000); f, myoglobulin (17,200); g, cytochrome c (12,500).

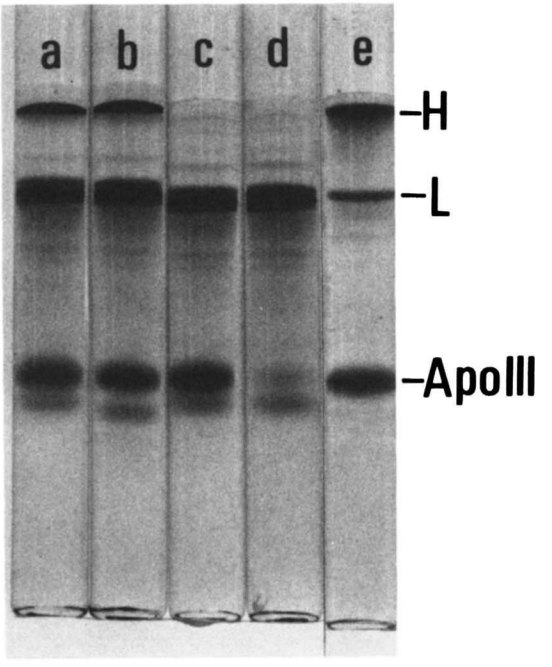

Fig. 7. SDS-polyacrylamide gel (7.5%) electrophoresis of hemolymph and the subnatant fraction from resting and AKH-injected male locusts. a, Resting hemolymph (20 μg of protein); b, AKH-injected hemolymph (10 pmol/animal, 90 min after injection, 20 μg of protein); c, subnatant fraction obtained after density gradient centrifugation of resting hemolymph (15 μg of protein); d, subnatant fraction obtained after density gradient centrifugation of AKH-treated hemolymph (15 μg of protein); e, activated lipophorin (15 μg of protein). H, heavy chain; L, light chain. The dense bands that appear near the light chain of lipophorin represent, at least in part, the subunit peptide (mol wt 83,000) of a biliverdin-binding cyanoprotein (17).

particles is confirmed by observations of native polyacrylamide gel electrophoresis (Fig. 4). These observations could be interpreted to suggest that the amount of diacylglycerol loaded onto lipophorin particles is variable. However, if this interpretation is correct, the activated lipophorin particles should show variable densities. As shown in Fig. 1, the peak of activated lipophorin is as sharp as the peak of resting lipophorin, thereby indicating that activated lipophorin is equivalent to resting lipophorin with regard to homogeneity in density. Thus, there appears to be a discrepancy between the heterogeneity in size and homogeneity in density of activated lipophorin particles.

In our earlier study (13), the molecular weight of resting lipophorin was determined by the sedimentation–equilibrium method and estimated to be about 600 K, of which 80 K may be attributed to the diacylglycerol content. The diacylglycerol content of activated lipophorin is almost three times greater than that of resting lipophorin (Table 1), thus the total molecular weight of the diacylglycerol component of activated lipophorin is increased by about 160 K. Scanning of the SDS-electropherogram of activated lipophorin in a Shimadzu chromatographic scanner (model, CS-900), assuming that the three apoproteins (heavy, light, and Apo-III) stain equally, suggests that eight Apo-III molecules become associated with the resting lipophorin to contribute to the production of activated lipophorin.[2] Thus, in addition to the diacylglycerol increase of 160 K, the molecular weight of activated lipophorin is increased by a further 160 K (20 K × 8) over that of resting lipophorin. Accordingly, the molecular weight of activated lipophorin should be about 920 K (600+160+160), assuming that each lipophorin molecule receives an equal amount of diacylglycerol from fat body in response to AKH-injection. The densities of activated and resting lipophorins are 1.065 and 1.120, respectively (Fig. 1 and text), therefore, the ratio of the theoretical volumes may be calculated:

$$\frac{V_{act}}{V_{rest}} = \frac{920/1.065}{600/1.120} = 1.61.$$

Accordingly, the ratio of diameters ($D \propto V^{1/3}$) may be calculated:

$$\frac{D_{act}}{D_{rest}} = \sqrt[3]{1.61} = 1.17.$$

Thus, given the mean diameter of resting lipophorin as 14.5 nm (Fig. 3), the diameter of activated lipophorin should be 14.5 × 1.17 = 17.0 nm. However, most of the lipophorin particles observed in the present study exceed this size and the largest particle (33.6 nm) is almost exactly double the calculated theoretical size. If a particle this size was the result of increased diacylglycerol content, the particle would have to contain at least 50 times more diacylglycerol than resting lipophorin and would, therefore, be a very low density particle. As indicated above, however, density gradient ultracentrifugation of activated lipophorin demonstrates homogeneity with regard to density and suggests that the density of each lipophorin particle is relatively uniform.

The explanation for the larger particles observed in the present study is not completely resolved but it is very possible that these particles result from fusion of primary activated lipophorin particles. In fact, the electron micrograph shows some images that may represent the process of such fusion (Fig. 2B, see arrow). If two molecules of the primary activated lipophorin are fused, the theoretically calculated diameter of the fused particle should become 1.26 times larger than that of the primary activated lipophorin; 17.0 × 1.26 = 21.4 nm which is close to the mean diameter (21.7 nm) observed for activated lipophorin on electron microscopy (Fig. 3B). Similarly, the largest particle (33.6 nm) may theoretically result from fusion of about

[2]This proposal has been confirmed recently by applying known amounts of purified Apo-III to SDS-polyacrylamide gel electrophoresis (unpublished results).

eight molecules of primary activated lipophorin. Irrespective of the size of the particles formed by fusion, the ratio of protein to lipid should remain the same for each fused particle, and this results in the homogeneity in density. The possible intermolecular fusion seems, at present, to be the only reasonable hypothesis that accounts for the discrepancy between the heterogeneity in size and the homogeneity in density of the activated lipophorin particles. It is not known whether such fusion occurs physiologically or whether it is an artefact of the experimental procedures employed in this study. Preliminary results suggest that the size of activated lipophorin particles is affected by experimental conditions, including the method by which hemolymph is collected. For example, the size distribution of particles prepared by the specific precipitation method differs from that of particles separated by density gradient centrifugation (Fig. 2 and text). The possible fusion of primary activated particles may also account for the appearance of extremely high molecular weight, diacylglycerol-containing lipoprotein fraction (fraction O) at a void volume on gel filtration of locust hemolymph (2). In fact, Wheeler, Mundy, and Goldsworthy (23) have observed, on the negatively stained electron micrograph, that the fraction O comprises large irregularly shaped aggregates. This aggregate may represent a product formed as a result of excessive intermolecular fusion as proposed in the present study.

The current study indicates that the transformation of resting lipophorin to activated lipophorin is reversible; the lipophorin activated by AKH returns to the original form within 24 hr after AKH injection. It is possible, however, that the activated lipophorins undergo complete breakdown and disappear from hemolymph within 24 hr, and that newly synthesized lipophorin molecules appear in hemolymph. Our recent study on the longevity of lipophorin in locust hemolymph indicates *1*) that the half-life of the protein moiety of lipophorin is about 5–6 days whereas that of the diacylglycerol component is only 2–3 hr, and *2*) that the half-life of the protein moiety is not affected by the injection of AKH (24). This observation supports the proposal that lipophorin, synthesized initially by the fat body and released into hemolymph, serves as a "reusable shuttle" to transport a variety of lipids between various tissues (9, 13); furthermore, the data negate the possibility that newly synthesized lipophorin comprises a major component of the hemolymph lipophorin pool within 24 hr of AKH injection.

The present study also indicates that the molecular weight of Apo-III from locust hemolymph is about 20,000 (Fig. 6), a figure that parallels the value of 20,000 reported for the C_2 protein of locust hemolymph (6). By contrast, Apo-III from hemolymph of *M. sexta* has a molecular weight of 17,000 and is present in the native state as a dimeric form (12). Thus, interspecific differences may occur in the nature of Apo-III between Orthopteran and Lepidopteran species.

Attempts to demonstrate an effect of AKH on lipophorin in vitro were unsuccessful and, therefore, the questions of how and under what conditions Apo-III associates with resting lipophorin remain unresolved. It is also not known how the association of Apo-III with resting lipophorin facilitates the loading of diacylglycerol from fat body in response to stimulation by AKH. These problems are currently under investigation in this laboratory.

This study was partly supported by a research grant (57480016) from the Japanese Ministry of Education, by the Japan Society for the Promotion of Science, and by the Natural Sciences and Engineering Research Council of Canada through the Canada-Japan Senior Scientist Exchange Programme.

Manuscript received 28 February 1985.

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