Biosynthesis of apolipophorin-III by the fat body in locusts

Susumu Izumi,* Kenji Yamasaki,* Shiro Tomino,* and Haruo Chino**

Department of Biology, Tokyo Metropolitan University,* Setagaya-ku, Tokyo, Japan, and Biochemical Laboratory Institute of Low Temperature Science,** Hokkaido University, Sapporo, Japan

Abstract Biosynthesis of locust apolipophorin-III (apo-III) was studied in vitro. Gel electrophoresis and immunoblotting analyses of the locust hemolymph demonstrated that apo-III first appears in the hemolymph on the day 3 of the adult stage after the final molt and its hemolymph concentration increases thereafter. When incubated in vitro in a medium containing radioactive amino acid, the fat body cells synthesized the radiolabeled apo-III and released it into the medium. The developmental change in the apo-III synthesizing activity in the fat body reflected that of the apo-III concentration in the hemolymph. RNA isolated from the adult fat body directed the synthesis of apo-III as a major translation product in a cell-free system. ▆▆ These results indicate that the fat body is the tissue responsible for the synthesis of the locust apo-III, and biosynthesis of apo-III is developmentally regulated at the level of mRNA in accordance with the flight activity of the locust.—**Izumi, S., K. Yamasaki, S. Tomino, and H. Chino.** Biosynthesis of apolipophorin-III by the fat body in locusts. *J. Lipid Res.* 1987. **28:** 667–672.

Supplementary key words lipophorin • diacylglycerol • insect adipokinetic hormone • hemolymph

Lipophorin is the major lipoprotein in the hemolymph of most insects (1), and serves as a reusable shuttle to transport various lipids including diacylglycerol, hydrocarbons, cholesterol, and carotenoids between tissues (2). Locust adipokinetic hormone (AKH), a blocked decapeptide (3), is released from the corpora cardiaca in response to flight (4) and promotes the association of non-lipid-containing protein, named apolipophorin III (5), with lipophorin and the loading of diacylglycerol from the fat body by lipophorin to produce larger, lower density lipophorin particles (6).

The purification of locust apolipophorin III (apo-III) from the hemolymph has recently been achieved in our laboratory and we have demonstrated the following points regarding the physicochemical properties of apo-III (7). *1*) There are three different molecular species of apo-III in the locust hemolymph. *2*) The three apo-IIIs, apo-III-a, apo-III-b, and apo-III-c, are indistinguishable with regard to the mobility on SDS-PAGE, amino acid composition, and immunodiffusion. However, they have different isoelectric points and, therefore, are separable on native- and urea-PAGE. *3*) The three apo-IIIs are glycoproteins containing fucose, mannose, and glucosamine. *4*) The native molecular weight of apo-III (ca. 19,500) is almost equivalent to the molecular weight (ca. 20,000) determined by SDS-PAGE, thereby indicating that the locust apo-III exists in hemolymph as a monomeric form. *5*) A total of 9 moles of apo-III (2 moles apo-III-a, 6 moles apo-III-b, and 1 mole apo-III-c) associate with each mole of lipophorin in response to the action of AKH.

Lipophorin itself is synthesized by the fat body and released into hemolymph (8) and always exists in the hemolymph throughout the life of the insect from the larval stage to the adult stage (2). However, the question of which tissue is responsible for the synthesis of apo-III remains unanswered. The flight of the locust is restricted to the adult stage and, therefore, it is also important to determine when the synthesis of apo-III is initiated in relation to the flight activity and the mechanism of AKH action.

In this report, we provide the evidence that, in locusts, the fat body is the tissue responsible for the synthesis of apo-III, and that the synthetic activity first appears at a certain stage of the adult, 3 days after the final molt. This study also reports the translation in a wheat germ cell-free system of mRNA for apo-III isolated from the fat body.

MATERIALS AND METHODS

Chemicals

L-[^{35}S]Methionine (sp act, 800 Ci/mmol) was obtained from Amersham. The biotinylated goat anti-rabbit IgG

Abbreviations: apo-III, apolipophorin-III; PAGE, polyacrylamide gel electrophoresis; AKH, adipokinetic hormone; TBS, Tris-buffered saline.

antibody and Vectastain ABC reagent were purchased from Vector Laboratories. Other chemicals used were purchased from commercial sources.

Animals and collection of hemolymph

Locusts, *Locusta migratoria,* were reared under crowded conditions and fed on prairie grass at 28°C with continuous light. Hemolymph was collected from larvae and adults by the flush method (9) and was diluted 20-fold with 20 mM Tris-HCl, 0.15 M NaCl, pH 7.5 (Tris-buffered saline, TBS) containing 10 mM EDTA. The diluted hemolymph samples were centrifuged at 10,000 g for 10 min to remove the hemocytes and stored at −80°C until use.

Preparation of antibody against apo-III

Locust apo-III was purified from hemolymph of the resting adult as described previously (7). Anti-apo-III antibody was prepared by subcutaneous injection on the back of a rabbit of the purified apo-III-b (major apo-III of the three apo-IIIs) emulsified with Freund complete adjuvant as described previously (7). Anti-apo-III IgG was separated from serum by ammonium sulfate precipitation at 40% saturation and dialyzed against TBS.

Incubation of fat body

Fat bodies were isolated from locusts into the sterilized locust medium (10) and rinsed three times with same medium. Approximately 100 μg of fat body was incubated in 50 μl of the locust medium containing 50 μCi [35S]methionine for 4 hr in an Eppendorf tube at 25°C. After incubation, fat bodies were withdrawn from the medium and homogenized with 0.5 ml of TBS. The culture medium was also diluted with 0.5 ml of TBS. The homogenates and the diluted media were centrifuged at 10,000 g for 10 min at 4°C. An aliquot (100 μl) of the supernatant was subjected to SDS-PAGE and the rest was used for immunoprecipitation analysis. In some experiments, fat body and medium were homogenized together without separation. The mixture was centrifuged as above and the supernatant was subjected to immunoprecipitation analysis.

Isolation of RNA

Fat bodies were isolated from the male locust 14 days after the last ecdysis and homogenized with ten volumes of 7 M guanidine–HCl containing 10 mM EDTA, pH 7.0. The homogenate was mixed with a half-volume of ethanol and kept at −20°C for 90 min. The precipitate was collected by centrifugation at 10,000 g for 10 min at 4°C. The pellet was dissolved in 7 M guanidine–HCl containing 10 mM EDTA, pH 7.0, and mixed with ethanol as above. The process of dissolution and precipitation was repeated three times. RNA was extracted from the pellet with sterilized water and the extract was shaken with an equal volume of CHCl3–*iso*-butanol 1:1 (v/v). After centrifugation at 3,000 g for 10 min at 4°C, the aqueous phase was made 2.5 M with respect to LiCl and kept at −20°C overnight to precipitate RNA. RNA was collected by centrifugation at 3,000 g for 30 min at 4°C and washed with ethanol. The final RNA preparation was dissolved in sterilized water and stored at −80°C.

Translation of RNA in vitro

A wheat germ fraction (S-30) was prepared according to the method of Roberts and Patterson (11) with some modification (12). The incubation mixture for cell-free protein synthesis contained the following components in a final volume of 50 μl: 30 mM HEPES–KOH, pH 7.5, 1 mM ATP, 0.2 mM GTP, 8 mM phosphocreatine, 5 μCi [35S]methionine, 0.4 mM each of 19 unlabeled amino acids, 0.6 mM spermidine-HCl, 1.5 mM magnesium acetate, 86 mM KCl, 1 mM dithiothreitol, 0.2 mg/ml of creatine kinase, 15 μl of wheat germ S-30, and RNA. The mixture was incubated at 22°C for 90 min.

Immunoprecipitation

At the end of fat body incubation, 200 μl of TBS was added to each culture and the mixture was homogenized. The homogenate was centrifuged at 10,000 g for 10 min at 4°C. An aliquot (150 μl) of supernatant was mixed with anti-apo-III IgG (150 μg) and incubated for 20 min at 37°C. Carrier apo-III (apo-III-b, 10 μg) was added to the mixture and incubation was continued for 30 min at 37°C and in the cold for 15 hr. The reaction mixture for cell-free protein synthesis was also diluted with 200 μl of TBS at the end of incubation and treated with antibody as above. The immunoprecipitates were collected by centrifugation and washed three times with TBS. The precipitates were dissolved in SDS-sample buffer (13) and subjected to SDS-PAGE.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was carried out as described by Laemmli (13) using 15% or 10–15% gradient acrylamide gel prepared in a slab gel electrophoresis apparatus. Protein samples were treated with SDS-sample buffer for 2 min in boiling water. Electrophoresis was performed at 25 mA constant current until tracking dye had reached the end of the gel. Proteins in the gel were stained with Coomassie blue.

Immunoblotting and detection of antigen

After electrophoresis, proteins were electroblotted onto nitrocellulose filter in 25 mM Tris-glycine (pH 8.3) in 20% methanol (v/v) at 0.2 A for 17 hr. After blotting the nitrocellulose filter was removed from the gel and washed with three changes of TBS, and incubated for 30 min in 20 ml of TBS containing 20% fetal calf serum. The antibody against the apo-III (50 μg) was added to the mixture
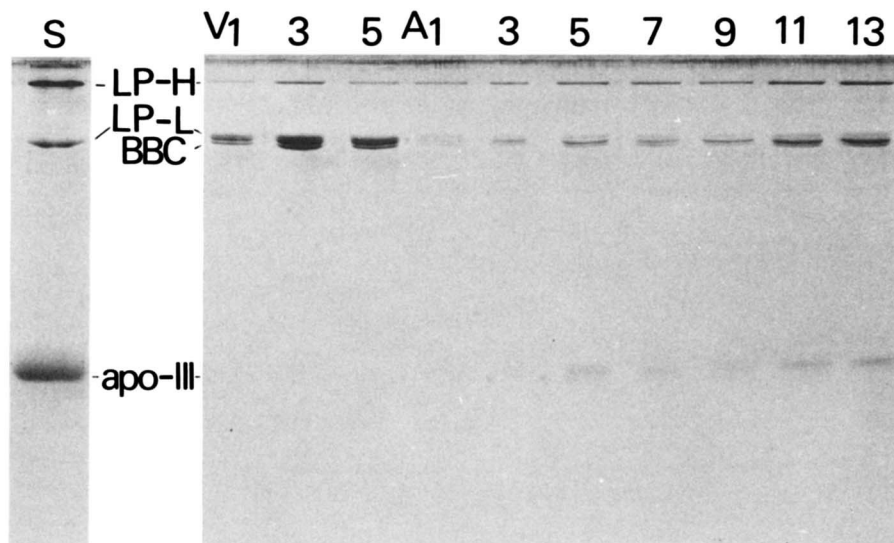
Fig. 1. Developmental changes in concentration of *L. migratoria* hemolymph proteins. Hemolymph was collected from the locusts at 48-hr intervals from one day after the fourth larval ecdysis. The hemolymph samples were diluted 20-fold with saline. Each 5 μl of the diluted hemolymph sample was electrophoresed in the Laemmli system using 15% polyacrylamide gel. Proteins were stained with Coomassie blue. V and A above the electrophoreogram represent the fifth instar and the adult stage, respectively. The numbers represent developmental stage in days. LP-H, LP-L, and BBC indicate lipophorin heavy chain, lipophorin light chain, and biliverdin-binding cyanoprotein (14), respectively; S, activated lipophorin as standard prepared from hemolymph of AKH-injected locust (6).
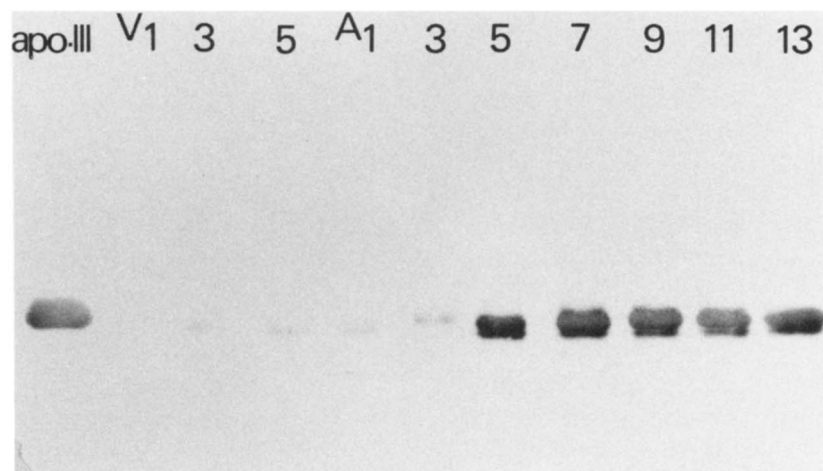

Fig. 2. Immunoblotting analysis of plasma proteins. The hemolymph samples were electrophoresed as described in Fig. 1. Immunoblotting was performed as described in Materials and Methods. V, A, and the numbers above the electrophoreogram represent developmental stages as indicated in Fig. 1. The purified apo-III (apo-III-b, 10 μg) was electrophoresed as standard in the lane of apo-III.

and incubation continued for 17 hr. After incubation the nitrocellulose filter was given three 10-min washes with TBS and then incubated with the biotinylated secondary antibody for 30 min with gentle agitation. The filter was washed with TBS three times (10 min each), treated with Vectastain ABC reagent for 30 min as suggested by the manufacturer, and then washed with TBS as above. Finally, the apo-III antigen was visualized by incubating the filter in peroxidase substrate solution containing 1 part 4-chloro-1-naphthol (3 mg/ml in methanol), 2 parts TBS, and 0.005 part H_2O_2 (30% aqueous solution) for 30 min.

Fluorography

After electrophoresis and staining for proteins, the polyacrylamide gel was treated with EN³HANCE (Dupont), dried, and fluorographed using Fuji RX film for 1 to 2 days at −80°C.

RESULTS AND DISCUSSION

Change of plasma proteins during fifth instar and adult stage

To investigate when the apo-III first appears in the hemolymph, the change of plasma protein composition was followed during development. After the fourth ecdysis, the hemolymph was collected from male locusts at 48-hr intervals and proteins were analyzed by SDS-PAGE. As seen in **Fig. 1,** the composition of proteins in the male locust is relatively simple and lipophorin heavy chain (apo-I, mol. wt. 250 K), lipophorin light chain (apo-II, mol. wt. 85 K) and biliverdin-binding cyanoprotein (mol. wt. 83 K) (14) are detected as major plasma proteins in the hemolymph at the fifth larval instar. A new protein having a molecular weight about 20 K appeared in addition to the above proteins 5 days after the final ecdysis and its concentration increased thereafter. This protein co-migrated with the purified apo-III as standard. To confirm whether the 20 K protein was identical to apo-III, an immunoblotting analysis of plasma protein was adopted by use of an antibody against apo-III. The result is shown in **Fig. 2** and clearly demonstrates that the 20 K protein is recognized by the antibody, thus confirming that the 20 K protein detected in adult hemolymph is identical to apo-III. Apo-III was barely detectable in the hemolymph before the third day of the adult period. The hemolymph concentration of apo-III rose sharply on the day 5. Thereafter, little change in the concentration of apo-III in hemolymph was noticed during the adult stage. These results essentially agree with those reported for the tobacco hornworm, *M. sexta* (5, 15); the apo-III of *M. sexta* is practically absent from the hemolymph during the larval

stage and it accumulates in large amounts during the adult stage.

Synthesis of apo-III in the cultured fat body

It is known that the majority of plasma proteins in insects are synthesized by the fat body and released into hemolymph. To ascertain the tissue synthesizing the locust apo-III, in vitro culture of fat body tissue was attempted. In the previous study we failed to detect methionine residues in the locust apo-III (7). However, careful re-examination of amino acid composition revealed that a slight but significant quantity of methionine is detectable in the acid hydrolyzate of apo-III. Therefore, apparent absence of methionine reported in our previous experiment would most likely be the consequence of degradative loss of this amino acid in the course of acid hydrolysis. In consideration of this fact and to increase the sensitivity of detection, the present experiments were performed with the use of [³⁵S]methionine, with a specific radioactivity that was highest among the commercially available radioactive amino acids.

The fat body was isolated from the male locust 13 days after the final ecdysis and incubated in the locust medium containing the radioactive methionine. After incubation,
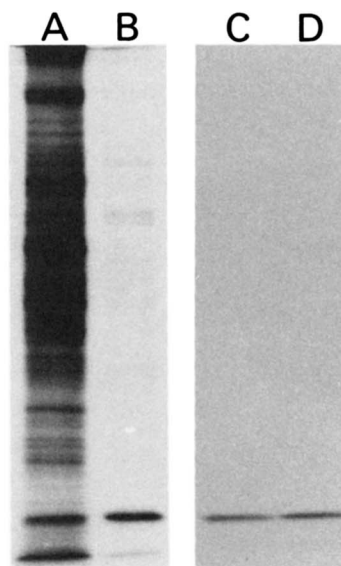

Fig. 3. SDS-PAGE of the proteins synthesized in the cultured fat body. The fat body was dissected out from male locusts on the day 13 of adult stage and was incubated at 25°C for 4 hr in a locust medium containing labeled methionine. After incubation, aliquots of fat body extract and medium were treated with anti-apo-III antibody, electrophoresed, and fluorographed as described in Materials and Methods. Total proteins synthesized by the fat body (A); proteins released in medium (B); and Immunoprecipitates from the extract of fat body (C), and from the medium (D).

proteins in the medium and those in the fat body cells were separately analyzed by SDS-PAGE and fluorography. As illustrated in **Fig. 3A**, electrophoresis revealed that a number of proteins, including a 20 K protein, were synthesized by the fat body. Among these, the 20 K protein was also detected as the major labeled protein in the medium (Fig. 3B). The extract of fat body and the culture medium were treated with the anti-apo-III antibody and the immunoprecipitates were analyzed in a similar fashion. Fig. 3 C and D demonstrates that the radioactive 20 K protein in both the fat body and the medium was reactive with anti-apo-III antibody. All these results reveal that apo-III is synthesized in the fat body cells from the adult locust and released into hemolymph.

For the study of the developmental change in the apo-III-synthesizing activity, the fat bodies were isolated from the locusts at intervals during development and were incubated in a medium containing radiolabeled methionine. After incubations, the immunoprecipitates from the fat body extracts were analyzed as above. Fluorography of the electrophoresed proteins indicated that the synthetic activity of apo-III was first detected on day 3 of the adult stage and increased thereafter **(Fig. 4)**. The biosynthetic activity of apo-III in the fat body cells reflects the developmental change in the hemolymph concentration of apo-III, and the biosynthesis of this plasma protein may be regulated in a stage-specific manner. The developmen-
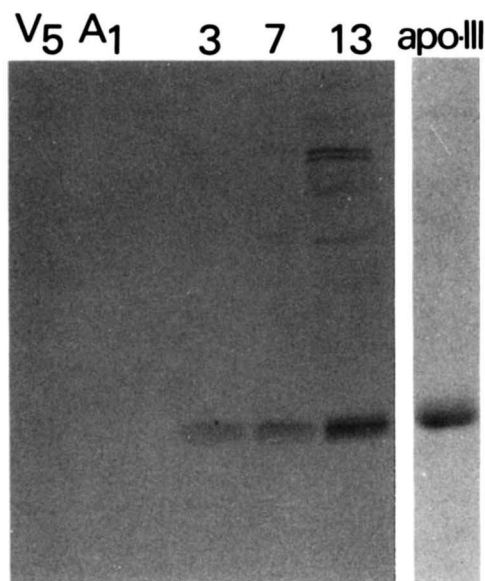

Fig. 5. SDS-PAGE of the translation products synthesized in vitro. Total fat body RNA (15 µg) isolated from the mature male was translated in a wheat germ cell-free translation system. After incubation for 90 min, total translation products (A) and immunoreactive translation products (B) were subjected to SDS-PAGE on 15% acrylamide gel and fluorographed as described in Materials and Methods.

Fig. 4. SDS-PAGE of immunoreactive proteins synthesized in the cultured fat body. Fat bodies were isolated from the locust on the day 5 of the fifth instar, days 1, 3, 7, and 13 of the adult stage (V5, A1, 3, 7, and 13) and were incubated in locust medium containing labeled methionine for 4 hr at 25°C. After incubation, the whole reaction mixture was homogenized and centrifuged. The extract was treated with anti-apo-III antibody, electrophoresed, and fluorographed as described in Materials and Methods.
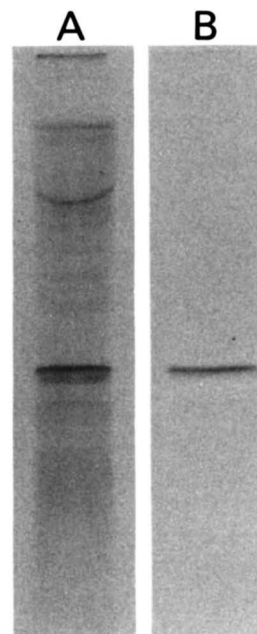
tal pattern of the apo-III-synthesizing activity seems physiologically meaningful in relation to the flight activity of the locust, since this insect can fly only at the adult stage.

Translation of fat body RNA

In a preliminary study of the regulation of apo-III biosynthesis, RNA was isolated from the fat bodies of male locusts on day 14 of the adult stage and translated in a wheat germ S-30 cell-free system in the presence of labeled methionine. Gel electrophoresis of the in vitro translation products demonstrated that the fat body RNA directs protein synthesis in a wheat germ cell-free system and a polypeptide having the same mobility as apo-III is synthesized as a major translation product **(Fig. 5A)**. For further identification, specific immunoprecipitation of the translation products was performed by use of an antibody against apo-III. As shown in Fig. 5B, the 20 K polypeptide was specifically precipitated by the antibody, showing that the mRNA coding for apo-III is present in the fat body cells of the adult locust.

We have recently achieved the cloning of mRNA sequence coding for apo-III and determined its partial nucleotide sequence. The primary structure of apo-III deduced from the nucleotide sequence also confirmed the occurrence of internal methionine residues (Ogino, K., S. Izumi, S. Tomino, and H. Chino, unpublished data).

Structural analysis of apo-III mRNA and the regulatory mechanism of apo-III synthesis is currently under investigation in our laboratory. ▮

Manuscript received 21 July 1986 and in revised form 31 December 1986.

REFERENCES

1. Chino, H., R. G. H. Downer, G. R. Wyatt, and L. I. Gilbert. 1981. Lipophorins, a major class of lipoprotein of insect hemolymph. *Insect Biochem.* **11:** 491.
2. Chino, H. 1985. Lipid transport: biochemistry of hemolymph lipophorin. *Insect Physiol. Biochem. Pharmacol.* **10:** 115–135.
3. Stone, J. V., W. Mordue, K. E. Batley, and H. R. Morris. 1976. Structure of locust adipokinetic hormone, a neurohormone that regulates lipid utilization during flight. *Nature.* **263:** 207–211.
4. Mayer, R. J., and D. J. Candy. 1969. Control of hemolymph lipid concentration during locust flight: an adipokinetic hormone from the corpora cardiaca. *J. Insect Physiol.* **15:** 611–620.
5. Kawooya, J. K., P. S. Keim, R. O. Ryan, J. P. Shapiro, P. Samaraweera, and J. H. Law. 1983. Insect apolipophorin III. *J. Biol. Chem.* **259:** 10733–10737.
6. Chino, H., R. G. H. Downer, and K. Takahashi. 1986. Effect of adipokinetic hormone on the structure and properties of lipophorin in locusts. *J. Lipid Res.* **27:** 21–29.
7. Chino, H., and M. Yazawa. 1986. Apolipophorin-III in locusts: purification and characterization. *J. Lipid Res.* **27:** 377–385.
8. Gellisen, G., and G. R. Wyatt. 1981. Production of lipophorin in the fat body of adult *Locusta migratoria:* comparison with vitellogenin. *Can. J. Biochem.* **59:** 648–654.
9. Chino, H., H. Katase, R. G. H. Downer, and K. Takahashi. 1981. Diacylglycerol-carrying lipoprotein of hemolymph of the American cockroach: purification, characterization and function. *J. Lipid Res.* **22:** 7–15.
10. Yamasaki, K. 1978. Selective protein-uptake by isolated insect oocyte. *Zool. Mag.* **87:** 416.
11. Roberts, B. E., and B. M. Patterson. 1973. Efficient translation of tobacco mosaic virus RNA and rabbit globin 9s RNA in a cell-free system from commercial wheat germ. *Proc. Natl. Acad. Sci. USA.* **70:** 2330–2334.
12. Izumi, S., S. Tojo, and S. Tomino. 1980. Translation of fat body mRNA from the silkworm, *Bombyx mori. Insect Bichem.* **10:** 429–434.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature. (London)* **227:** 680–685.
14. Chino, H., Y. Abe, and K. Takahashi. 1983. Purification and characterization of a biliverdin-binding cyano-protein from the locust hemolymph. *Biochim. Biophys. Acta.* **748:** 109–115.
15. Shapiro, J. P., and J. H. Law. 1983. Locust adipokinetic hormone stimulates lipid mobilization in *Manduca sexta. Biochem. Biophys. Res. Commun.* **115:** 924–931.