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Lipoprotein Biosynthesis in the Larvae of the Tobacco Hornworm, *Manduca sexta**

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Lipoprotein biosynthesis in larvae of the tobacco hornworm (*Manduca sexta*) was investigated. By immunoblotting, it was shown that the apoproteins are present in the fat body, but not in the midgut. Fat body incubated *in vitro* with [^{35}S]methionine secreted labeled apoproteins. However, when the density of the secreted particle was determined, it was found at 1.24–1.28 g/ml instead of 1.15 g/ml, which is the density of the circulating lipoprotein. Lipid analysis of immunoprecipitated lipoprotein secreted by the fat body showed a phospholipid/diacylglycerol ratio of 8.3 rather than 0.9, the ratio found in the circulating lipoprotein. When labeled oleic acid or triolein was fed to larvae, it was found that >98% of the label in the circulating lipoprotein was in diacylglycerol. In studies using animals raised on a fat-free diet, it was shown that the circulating lipoprotein has properties comparable to those of the material secreted *in vitro* by the fat body and that this diacylglycerol-poor particle can be converted to the normal lipoprotein by feeding a bolus of triolein. These data support the hypothesis that the fat body makes and secretes a "nascent" lipoprotein which contains apoproteins and phospholipid, but is devoid of diacylglycerol. The diacylglycerol is then picked up from the midgut to complete assembly of the mature circulating lipoprotein.

Lipids are transported in insect hemolymph by a high density lipoprotein called lipophorin (1–3). Although several papers have appeared describing the properties of lipophorins from a number of insects, relatively little is known about the biosynthesis of the lipoprotein. Two rather indirect studies have suggested that the site of synthesis is the fat body in locust (4, 5). However, no direct demonstration of lipophorin secretion by the fat body or characterization of the secreted material has been described.

In this paper, we will be concerned with lipoprotein biosynthesis in the fifth instar larvae of the tobacco hornworm, *Manduca sexta*. The lipoprotein present during the larval stage is called high density lipophorin-larval (HDLp-L)[1] and has been extensively studied (6, 7). HDLp-L contains 37% lipid, mainly phospholipid and diacylglycerol, and 63% protein (6). The protein moiety is comprised of two apolipoproteins: apolipophorin I (apoLp-I, M_r = 250,000) and apolipophorin II (apoLp-II, M_r = 80,000).

We shall show that lipoprotein biosynthesis in *M. sexta* larvae is unique, when compared to mammalian lipoprotein biosynthesis, in that the proteins and phospholipid are synthesized and assembled into a "nascent" particle in the fat body, which after secretion into the hemolymph picks up diacylglycerol from the midgut.

MATERIALS AND METHODS

Animals were raised as previously described (6). In some cases, animals were raised on a fat-free diet which was prepared as follows. The wheat germ component of the diet, which contains nearly all the lipid in the diet, was extracted in a Soxhlet apparatus with chloroform/methanol (3:1) overnight. After air drying, the extracted wheat germ was dried *in vacuo*. The lipid-free wheat germ was thoroughly mixed with sufficient cholesterol and methyl linolenate (Nu Chek Prep, Inc., Elysian, MN) to provide 0.05% by weight of each in the final diet and then used to prepare diet in the usual way. Animals were placed onto the fat-free diet at the beginning of the fourth instar. In order to isolate the hemolymph lipoprotein present in animals raised on the fat-free diet, hemolymph was collected in the middle of the fifth instar and subjected to density gradient centrifugation (8), except that the overlaying solution was a KBr solution of density 1.21 g/ml. When the gradient was fractionated (9) and the proteins were analyzed by SDS-PAGE (8), most of the apoproteins were found at densities between 1.24 and 1.28 g/ml rather than at 1.15 g/ml, the normal density for HDLp-L. Therefore this material will be called very high density lipophorin (VHDLp). In order to remove contaminating hemolymph proteins, the density gradient centrifugation procedure of Haunerland and Bowers (10) was employed, in which the overlaying solution is a KBr solution of density 1.23 g/ml.

Methods for lipoprotein compositional analysis have been described (6, 11), except that for hydrocarbon and cholesterol analysis, we employed 3% OV 101 on 100/120 mesh Gas-chrom Q (Applied Science Laboratories, Deerfield, IL) and a temperature program from 130 to 330 °C at 4 °C/min. Diheptadecanoylphosphatidylcholine (Avanti Polar Lipids, Inc., Birmingham, AL) was used as an internal standard for phospholipid determination. Methods for SDS-PAGE and immunoblotting have been described (6, 8). [9,10-^{3}H]Oleic acid, [^{3}H]triolein, [^{35}S]methionine were from New England Nuclear. Radioactive protein standards for SDS-PAGE were from Bethesda Research Laboratories. All other chemicals were reagent grade.

In Vivo Labeling Experiments—Five μCi of either [^{3}H]oleic acid or [^{3}H]triolein was applied to a small portion of diet. Animals were then fed the diet. At various times after consumption of the diet, hemolymph was collected for lipoprotein isolation (8). The lipids were extracted by the method of Bligh and Dyer (13) and separted by thin layer chromatography. Areas corresponding to each lipid were scraped from the plate and counted using liquid scintillation spectrometry.

Triacylglycerol Feeding to Animals on Fat-free Diet—In order to determine whether a bolus of triacylglycerol fed to animals on a fat-free diet could cause conversion of VHDLp to HDLp-L, the following experiment was conducted. Ten animals raised on the fat-free diet were fed 50 μl of triolein by instilling the material directly into the midgut through a piece of PE-10 polyethylene tubing (Clay Adams, Parsippany, NJ). Six h later, these animals and 10 control animals

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[1] The abbreviations used are: HDLp-L, high density lipophorin-larval; VHDLp, very high density lipophorin; apoLp-I, apolipophorin I; apoLp-II, apolipophorin II; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

raised on the fat-free diet but not fed triolein were bled, and the hemolymph was subjected to density gradient centrifugation (8). The gradient was fractionated into 2-ml fractions starting from the top. An aliquot of each fraction was analyzed by SDS-PAGE. The gels showed that the apoproteins were found in two density ranges such that high density lipophorin was found in fractions with densities < 1.185 g/ml and VHDLp in fractions of densities > 1.185 g/ml. Therefore, the fractions were combined on this basis. The two pooled fractions were analyzed for lipid and fatty acid composition.

In Vitro Lipoprotein Synthesis—Fat body was dissected from Day 2 fifth instar larvae and rinsed thoroughly with phosphate-buffered saline (0.1 M sodium phosphate, pH 7.0, 0.15 M NaCl). The total fat body from each animal was incubated for 1 h in 0.5 ml of Grace's insect medium (Gibco Laboratories) without methionine, containing 0.03 mg/ml penicillin, 0.1 mg/ml streptomycin, and 25 μCi of [^{35}S] methionine at 30 °C with gentle shaking. At the end of the incubation, the fat body was removed, and the medium was filtered through a pad of glass wool and made 1 mM in diisopropylphosphofluoridate. Lipoproteins in the medium were precipitated with rabbit antibody to HDLp-L attached to immunobeads (Bio-Rad) after an initial precipitation with nonimmune rabbit serum and *Staphylococcus aureus* cells to remove nonspecific precipitating material (12). The immunoprecipitate was collected by centrifugation and washed three times with 50 mM Tris, pH 7.4, containing 0.15 M NaCl, 5 mM EDTA, 2 mg/ml bovine serum albumin, 0.02% sodium azide, and 0.1% Triton X-100 and then dissolved in SDS sample buffer and subjected to SDS-PAGE. Labeled proteins were visualized by fluorography by soaking the gel in a solution containing 2% β-methylnaphthalene and 0.4% 2,5-diphenyloxazole in glacial acetic acid, drying *in vacuo*, and exposing to Kodak XAR-5 film overnight at −70 °C.

In order to characterize lipid secretion by the fat body *in vitro*, the same type of incubation was conducted except that the labeled amino acid was replaced with 1 μCi of [^{3}H]oleic acid. The incubation medium was processed as above except that the nonspecific immunoprecipitation was omitted. The immunoprecipitate was resuspended in phosphate-buffered saline and extracted by the method of Bligh and Dyer (13). The lipid extract was mixed with unlabeled diacylglycerol and phospholipid, which were then separated by thin layer chromatography; and the lipids were extracted, counted, and analyzed (6). In three preliminary experiments, it was determined that the ratio of the specific activity of diacylglycerol to phospholipid was 1.05 ± 0.05; therefore, a comparison of the total counts in each lipid gives an accurate measure of the amount of each lipid secreted by the fat body.

RESULTS

In preliminary experiments, we found by immunoblotting that a homogenate of fat body contained easily detectable amounts of apoLp-I and -II, but that these apoproteins could not be detected in homogenates of midgut. Therefore, we concentrated on fat body as the site of lipoprotein biosynthesis. When fat body was incubated *in vitro* with labeled methionine and the incubation medium, which contains secreted proteins, was immunoprecipitated with antibody raised against HDLp-L, it was clear that the fat body synthesized and secreted both apoLp-I and -II, as shown in Fig. 1.

When the incubation medium was subjected to density gradient centrifugation, the labeled apoproteins were found in the density range of 1.24–1.28 g/ml, not at 1.15 g/ml as would be expected for HDLp-L (6). In HDLp-L, the molar ratio of phospholipid to diacylglycerol is 0.9 (6); however, the ratio found in the immunoprecipitate was 8.3 ± 1.2 (n = 3). Thus, the lipoprotein secreted by the fat body contains apoLp-I and -II but a much higher ratio of phospholipid to diacylglycerol than is found in HDLp-L. The high content of phospholipid and low content of diacylglycerol would be consistent with the observed density. Because of the small amount of material secreted, attempts to purify the secreted lipoprotein were unsuccessful, but an analogous lipoprotein from the hemolymph of animals raised on the fat-free diet has been characterized (see below).

Since the lipoprotein secreted by the fat body was relatively devoid of diacylglycerol, we considered the possibility that the midgut might be the source of the diacylglycerol found in HDLp-L. To test this hypothesis, we fed either labeled oleic acid or triolein to animals and then isolated HDLp-L 15 min to 3 h after feeding. The distribution of label among the lipids in HDLp-L is shown in Table I. Identical results were obtained with either labeled oleic acid or triolein, and there was not a significant change in the distribution over the time period studied. These data show that more than 95% of the label appearing in HDLp-L after an oral feeding is found in diacylglycerol with less than 2% found in phospholipid or other lipids. Thus, these data strongly supported the hypoth-

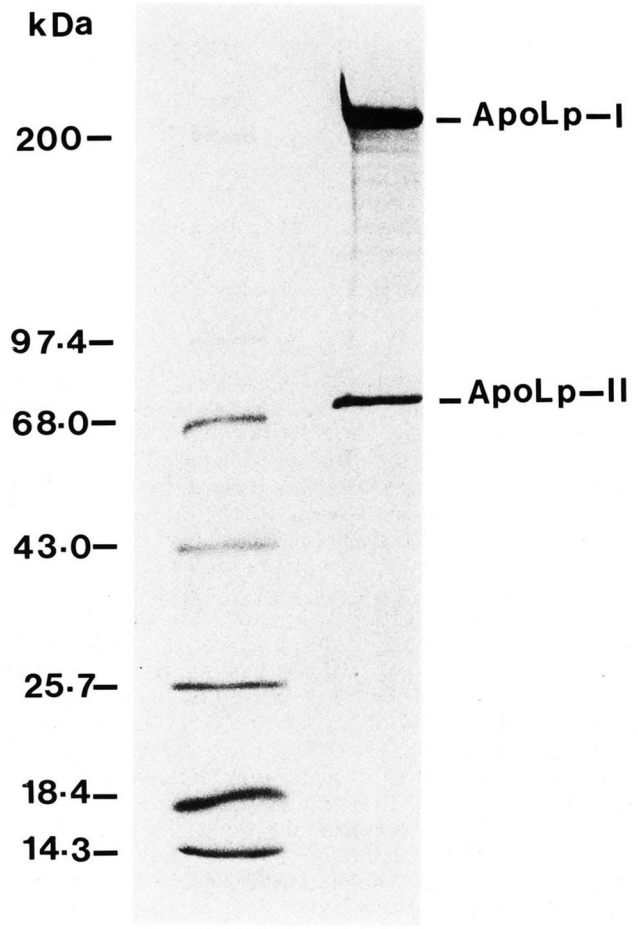

FIG. 1. **SDS-PAGE of apoproteins immunoprecipitated from the medium when fat body was incubated *in vitro* with [^{35}S]methionine in Grace's medium.** The *left lane* contains molecular mass markers. The *right lane* contains the redissolved immunoprecipitate with the positions of apoLp-I and apoLp-II indicated.

TABLE I

Distribution of [^{3}H]oleic acid among the lipids of HDLp-L after feeding labeled triolein or oleic acid

Animals were fed 5 μCi of either ^{3}H-labeled oleic acid or triolein, and HDLp-L was isolated 15, 30, 60, 120, or 180 min later. The lipids were separated by thin layer chromatography, and the amount of label in each was determined. Since there was no significant difference between feeding oleic acid or triolein nor a significant change with time, the data have been combined in this table and represent the mean ± S.D. for eight determinations.

Lipid	% of label
Diacylglycerol	95.8 ± 0.9
Phospholipid	1.5 ± 0.3
Free fatty acid	1.7 ± 1.4
Triacylglycerol	0.3 ± 0.3
Monoacylglycerol	0.7 ± 0.2

TABLE II

Composition of the lipoprotein isolated from the hemolymph of fifth instar larvae raised on a fat-free diet

The data represent the mean ± S.D. for four preparations.

Component	Weight %
Phospholipid	8.8 ± 2.7
Diacylglycerol	2.6 ± 1.2
Cholesterol	0.2 ± 0.1
Hydrocarbon	0.9 ± 0.4
Triacylglycerol	Trace
Protein	87.5 ± 2.4

esis that the diacylglycerol in HDLp-L arises from the midgut.

The hypothesis that the fat body secretes a diacylglycerol-poor, phospholipid-rich nascent lipoprotein was supported by studies using animals raised on a fat-free diet. When the hemolymph from such animals was subjected to density gradient centrifugation, it was noted that most of the apoproteins were found in the density range of 1.24–1.28 g/ml, with the highest concentration at a density of 1.255 g/ml. The composition of VHDLp is shown in Table II. The properties of this lipoprotein are consistent with those reported above for the nascent lipoprotein secreted *in vitro* by the fat body. The fact that VHDLp contains essentially no diacylglycerol coupled with the fact there is no lipid in the diet lends support to the suggestion that the diacylglycerol found in HDLp-L arises from processing of dietary lipid by the midgut.

Additional support for this hypothesis comes from the following experiment. Animals raised on the fat-free diet were fed a bolus of triolein, and after 6 h, hemolymph was collected and the lipoproteins were fractionated by density gradient centrifugation. The gradient was divided into two fractions: density < 1.185 g/ml and density > 1.185 g/ml. The animals on the fat-free diet had 170 μg of diacylglycerol/ml of hemolymph with 56% in the density > 1.185 fraction and 44% in the density < 1.185 fraction. In addition, 67% of the phospholipid was in the density > 1.185 fraction and 33% in the density < 1.185 fraction. The ratio of oleate to palmitate in the diacylglycerol in each fraction was 1.3. After feeding triolein, the hemolymph contained 505 μg of diacylglycerol/ml with 70% in the density < 1.185 fraction and 30% in the density > 1.185 fraction and with 57% of the phospholipid in the density < 1.185 fraction and 42% in the density > 1.185 fraction. The ratio of oleate to palmitate in the diacylglycerol in the density < 1.185 fraction was 9.1, and in the density > 1.185 fraction it was 7.0. The ratio of oleate to palmitate in the phospholipid in each fraction was 7.6 ± 0.3 and was not affected by triolein feeding. These data show that feeding triolein to animals on a fat-free diet causes an increase in the diacylglycerol content of hemolymph, that most of this increase is due to diolein derived from the fed triolein (as judged by the ratio of oleate to palmitate), and that the increased diolein in the hemolymph causes a significant shift of lipoprotein from the density > 1.185 fraction to the density < 1.185 fraction.

DISCUSSION

The data presented in this paper support the following hypothesis for lipoprotein biosynthesis and assembly in larvae of *M. sexta*. The apoproteins are synthesized in the fat body where they associate with phospholipid to form a nascent VHDLp which is essentially devoid of diacylglycerol. The VHDLp is then secreted from the fat body into the hemolymph. In the absence of dietary lipid, this is the predominant circulating lipoprotein. In animals on a normal diet, the circulating VHDLp then interacts with the midgut to pick up diacylglycerol which is derived from dietary lipid. The mechanism whereby diacylglycerol moves from the midgut into the hemolymph where it can become associated with the lipoprotein is unknown at present, but may involve the lipid transfer protein which has been purified from *M. sexta* hemolymph and partially characterized (14).

There are several points of difference between lipoprotein biosynthesis in *M. sexta* and mammals. Dietary lipid is carried by a high density lipoprotein in the insect, whereas in mammals dietary lipid is transported by chylomicrons. In mammals, chylomicron synthesis is coupled to fat intake into the gut, and the lipoprotein is synthesized in the mucosal cells of the gut. In insects, the lipoprotein is not synthesized in the midgut, but rather in the fat body, and lipoprotein biosynthesis is not coupled to fat intake. The secretion of a nascent lipoprotein by the fat body is somewhat analogous to the secretion of a nascent high density lipoprotein by the mammalian liver. However, maturation of the two nascent particles to the circulating form is quite different. In mammals, high density lipoprotein is produced by enzymatic modifications which occur in plasma, whereas high density lipophorin is produced by uptake of diacylglycerol from the midgut.

Although the major function of the lipoprotein during larval growth may be transport of dietary lipid to storage sites in the fat body, the fat body seems capable of synthesizing adequate amounts of storage lipid from dietary carbohydrate.[2] During adult development, the lipoprotein seems to function in transporting lipid from the lipid stores in the fat body to developing tissues. However, since lipoprotein biosynthesis does not occur during the pupal stage (6), the ability to transport lipid during adult development is entirely dependent on lipoprotein synthesis which occurred in the larval stage. Thus, whereas it may not be critical for the lipoprotein to be present during the larval stage in order to deposit fat stores, it must be made during this stage in order to function during adult development in the pupal stage. This may explain why lipoprotein biosynthesis and dietary fat intake are uncoupled in the larva.

REFERENCES

1. Chino, H., Downer, R. G. H., Wyatt, G. R., and Gilbert, L. I. (1981) *Insect Biochem.* **11,** 491
2. Chino, H. (1985) in *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* (Kerkut, G. A., and Gilbert, L. I., eds) Vol. 10, pp. 115–136, Pergamon Press, Ltd., Oxford
3. Beenakkers, A. M. Th., Van der Horst, D. J., and Van Marrewijk, W. J. A. (1985) *Prog. Lipid Res.* **24,** 19–67
4. Harry, P., Pines, M., and Applebaum, S. W. (1979) *Comp. Biochem. Physiol. B Comp. Biochem.* **63,** 287–293
5. Gellissen, G., and Wyatt, G. R. (1981) *Can. J. Biochem.* **59,** 648–654
6. Prasad, S. V., Ryan, R. O., Law, J. H., and Wells, M. A. (1986) *J. Biol. Chem.* **261,** 558–562
7. Pattnaik, N. M., Mundall, E. C., Trambusti, B. G., Law, J. H., and Kézdy, F. J. (1979) *Comp. Biochem. Physiol. B Comp. Biochem.* **63,** 469–476
8. Shapiro, J. P., Keim, P. S., and Law, J. H. (1984) *J. Biol. Chem.* **259,** 3680–3685
9. Ryan, R. O., Prasad, S. V., Henriksen, E. J., and Law, J. H. (1986) *J. Biol. Chem.* **261,** 563–568
10. Haunerland, N. H., and Bowers, W. S. (1986) *Biochem. Biophys. Res. Commun.* **134,** 580–586
11. Fernando-Warnakulasuriya, G. J. P., Staggers, J. E., Frost, S. C., and Wells, M. A. (1981) *J. Lipid Res.* **22,** 668–674
12. Harnish, D. G., Wyatt, G. R., and White, B. N. (1982) *J. Exp. Zool.* **220,** 11–19
13. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
14. Ryan, R. O., Wells, M. A., and Law, J. H. (1986) *Biochem. Biophys. Res. Commun.* **136,** 260–265

[2] G. J. P. Fernando-Warnakulasuriya and M. A. Wells, unpublished observation.