THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 259, No. 6, Issue of March 25, pp. 3680-3685, 1984
Printed in U.S.A.

Structural Studies on Lipophorin, an Insect Lipoprotein*

(Received for publication, February 15, 1983)

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An insect high density lipoprotein, lipophorin, can be rapidly isolated from larval *Manduca sexta* (tobacco hornworm) hemolymph by single vertical spin density gradient ultracentrifugation. The two apolipoproteins (M_r = 245,000 and 78,000; designated apoLp-I and apoLp-II, respectively) were readily dissociated and separated in 6 M guanidine HCl by gel permeation chromatography. ApoLp-I and apoLp-II showed no immunological cross-reactivity on electrophoretic blots of sodium dodecyl sulfate-polyacrylamide gels. ApoLp-I and apoLp-II from lipophorin of adult *M. sexta* behaved identically to their larval counterparts. Amino acid compositions of larval apoLp-I and apoLp-II were similar except with respect to tryptophan and cysteine; apoLp-I contained 32 residues/mol of tryptophan (1.5 mol %) and 22 residues/mol (1.1 mol %) of cysteine; apoLp-II contained 2 residues/mol of tryptophan (0.2 mol %) and 14 residues/mol of cysteine (2.1 mol %). In double immunodiffusion tests, antiserum against apoLp-I or whole lipophorin strongly precipitated lipophorin, while antiserum against apoLp-II caused only minor precipitation. This indicates relatively greater exposure of apoLp-I to the aqueous environment.

Unlike mammalian blood, insect hemolymph contains a single major class of low- to high-density lipoproteins, called lipophorins because of their role in lipid transport (Chino *et al.*, 1981a). Lipophorins can reversibly load lipid, increasing in size and decreasing in density from the high to the low density lipoprotein class upon association with a small soluble protein (Mwangi and Goldsworthy, 1977, 1981; van der Horst *et al.*, 1981; Wheeler and Goldsworthy, 1983a, 1983b; Shapiro and Law, 1983). Lipophorin appears to be the major carrier of hydrophobic natural products (Gilbert and Chino, 1974; Katase and Chino, 1982) and xenobiotics (Winter *et al.*, 1975; Skalsky and Guthrie, 1975; Fell *et al.*, 1976) in insect hemolymph. Though present in all life stages, lipophorin usually has been isolated from hemolymph of pupal and adult insects (Chapman, 1980). Recently, it was isolated and characterized from the hemolymph of larval *Manduca sexta* (Pattnaik *et al.*, 1979).

Compared to mammalian lipoproteins, lipophorins are unusual in both lipid and apoprotein content. Lipids, which constitute 39 to 48% of lipoprotein mass, consist of 33 to 56% diacylglycerol, 19 to 36% phospholipid, and 5 to 6% unesterified cholesterol (Chapman, 1980). Furthermore, lipophorins contain two relatively large apoproteins (M_r = 250,000 and ~80,000). In contrast, mammalian lipoproteins contain little diacylglycerol and a much higher proportion of nonpolar lipid (primarily triacylglycerol and cholesterol esters). Most mammalian lipoproteins also contain numerous small (M_r = <40,000) apoproteins, with the exception of the low density lipoproteins. These compositions suggest a difference in structural organization between mammalian and insectan lipoproteins.

Little is known about the structure of native lipophorin. Improved methods of lipophorin and apoprotein isolation from larval *M. sexta* reported here permit structural studies using immunological probes. We also report complete amino acid compositions of the two apoproteins, since cysteine and tryptophan contents were not previously reported (Pattnaik *et al.*, 1979). The marked similarity in amino acid content of the apoproteins indicated possible homology, and a recent report on locust lipophorin (Gellissen and Wyatt, 1981) suggested that a large apoprotein, not consistently present in lipophorin preparations, represented an aggregate of small apoproteins. We have, therefore, compared the apoproteins of *M. sexta* lipophorin, which we designate apoLp-I[1,2] (M_r = 245,000) and apoLp-II (M_r = 78,000), and demonstrate unequivocally that they are not homologous.

EXPERIMENTAL PROCEDURES[3]

RESULTS

Isolation of Lipophorin—Flotation in a KBr gradient using a single vertical spin at 50,000 rpm yielded a highly purified preparation of lipophorin, as judged by SDS-polyacrylamide slab gel electrophoresis (Fig. 4). Fig. 2 illustrates the efficiency of separation of lipophorin from the major hemolymph protein manducin (Kramer *et al.*, 1980), whose two subunits (M_r = 78,000 and 72,000) appear in fractions 14–20. A small amount of manducin was carried over into lipophorin fractions when tubes were fractionated from the bottom (Fig. 2). However, for routine preparations, the pure lipophorin was withdrawn

* This study was supported by Grant GM 29238 from the National Institute of General Medical Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: apoLp-I, apolipophorin I; apoLp-II, apolipophorin II; apoLp-III, apolipophorin III; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline.

[2] We have used Roman numerals to designate these insect apolipoproteins to avoid premature comparison to the lettered mammalian apolipoproteins.

[3] Portions of this paper (including "Experimental Procedures," Figs. 1–3, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-369, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

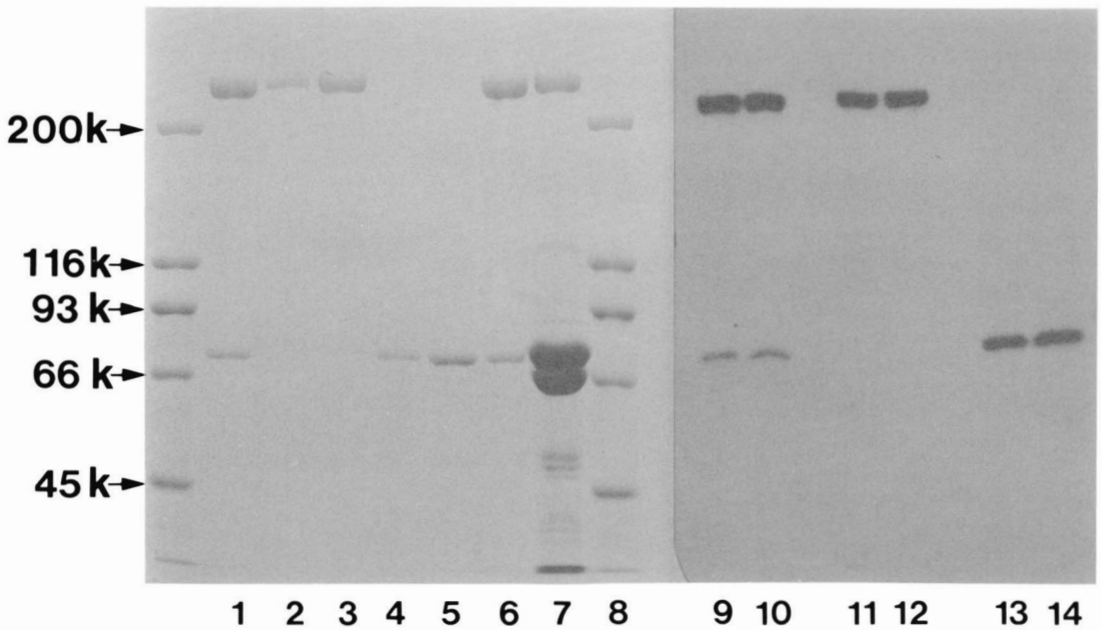

FIG. 4. **SDS-polyacrylamide gel electrophoresis and immunological cross-reactivity of lipophorin and apoproteins from *M. sexta*.** Whole hemolymph (*lanes 7, 10, 12, 14*), isolated lipophorin (*lanes 1, 6, 9, 11,* and *13*) or lyophilized apoLp-I (*lanes 2* and *3*) or apoLp-II (*lanes 4* and *5*) or standards (*left lane* and *lane 8*) were dissolved in sample buffer containing SDS, β-mercaptoethanol, and glycerol by heating in boiling H_2O for 3–5 min. Samples were applied to a 3–8% gradient SDS-polyacrylamide slab gel and subjected to electrophoresis. The *left lane* and *lanes 1–8* were separated from *lanes 9–14* and stained. *Lanes 9–14* were electrophoretically transferred to nitrocellulose for 18 h at 16 °C. After transfer, *lanes 9* and *10* were reacted with anti-lipophorin, *lanes 11* and *12* with anti-apoLp-I, and *lanes 13* and *14* with anti-apoLp-II. Immunoglobulins were labeled by reacting blots with *Staphylococcus* ^{125}I-protein A and located by autoradiography. Approximately 5–10 μg of protein per lane were applied to the *left lane* and *lanes 1–8*, 0.5 μg to *lanes 9–14*. Standards are: myosin (200,000), β-galactosidase (116,250), phosphorylase *b* (92,500), bovine serum albumin (66,200), and ovalbumin (45,000).

through the side of the tube with a hypodermic syringe (see Fig. 4, *lanes 1* and *6*). Recentrifugation of isolated lipophorin and comparison with a density gradient reference tube yielded a buoyant density of 1.13 g/ml (Fig. 1). Gel permeation HPLC demonstrated a single major peak, with a small amount (<10%) appearing at the void volume as aggregated material.

SDS-Polyacrylamide Gel Electrophoresis: Molecular Weight Determination and Carbohydrate Content—Earlier molecular weight determinations on SDS-polyacrylamide gels were made by comparison to cross-linked standards (Pattnaik *et al.*, 1979). Our more recent experience with these standards has been less satisfactory than with non-cross-linked standards. We, therefore, slightly revised our molecular weight estimates, using non-cross-linked standards (Bio-Rad high molecular weight standards, plus ferritin (220,000) and thyroglobulin (330,000), not shown) on 3–8% SDS-polyacrylamide gradient slab gels (Fig. 4). Molecular weights of apoLp-I and apoLp-II were calculated as 245,000 ± 7,000 and 78,000 ± 3,000 (M_r ± S.D., $n = 6$).

Scanning densitometry of stained apoproteins yielded an approximate apoLp-I:apoLp-II ratio of 3:1 by weight, corresponding to the 1:1 molar ratio previously observed (Pattnaik *et al.*, 1979). The stoichiometric ratio was further confirmed by separation of the two apoproteins by preparative SDS-gel electrophoresis. The fractions containing the two apoproteins were identified by analytical SDS-slab gel electrophoresis and the amounts of protein present in pooled fractions of each apoprotein were determined quantitatively by the Folin-phenol method of Peterson (1983), using SDS solutions of the pure apoproteins as standards. In two runs, apoLp-I/apoLp-II molar ratios of 1:13 and 0.87 were determined.

After separation on slab gels, both apoLp-I and apoLp-II stained with periodate-Schiff reagent, and both bound fluorescein-labeled concanavalin A.

Amino Acid Composition and UV Absorbance—Apoproteins were analyzed for content of amino acids, including tryptophan and cysteine (Table II). Spectrophotometric determination of tryptophan yielded values of 29 and 2 residues/mol of protein in apoLp-I and apoLp-II, respectively, compared to 32 and 2 residues/mol when determined by mild hydrolysis and chromatographic analysis. Specific absorbances for apoLp-I and apoLp-II at 280 nm in 6 M guanidine HCl were 0.716 mg^{-1} ml^{-1} and 0.300 mg^{-1} ml^{-1}, respectively. A visible wavelength scan of native lipophorin in PBS revealed a maximum absorbance at 455 nm (due to the presence of carotenoids) and profile identical to that observed in *Locusta migratoria* lipophorin (Peled and Tietz, 1975).

Immunological Comparison of Apoproteins—Immunological reaction of nitrocellulose-bound proteins was used to determine whether the apoproteins have common structural features. Lipophorin was subjected to electrophoresis on SDS-polyacrylamide slab gels, transferred electrophoretically to nitrocellulose, and reacted with antisera specific to whole lipophorin or to either apoprotein. IgG-bound proteins were located by reacting blotted proteins with *Staphylococcus* ^{125}I-protein A followed by autoradiography. Clearly, the anti-lipophorin showed immunoreactivity to both apoLp-I and apoLp-II (Fig. 4). However, anti-apoLp-I did not bind to apoLp-II, and anti-apoLp-II did not bind to apoLp-I. ApoLp-I and apoLp-II were the only proteins in fifth instar larval hemolymph that reacted with anti-lipophorin.

Lipophorin was isolated from adult *M. sexta* hemolymph and analyzed in the same manner, using antisera against larval lipophorin and apoproteins (Fig. 5). ApoLp-I and

TABLE II

Amino acid composition of lipophorin apoproteins

Amino acid	ApoLp-I		ApoLp-II	
	Residues/mol[a]	Mol %	Residues/mol[a]	Mol %
Aspartate[b]	232	11.2	73	11.0
Threonine	93	4.5	20	3.1
Serine	144	6.9	63	9.6
Glutamate[b]	199	9.6	67	10.2
Proline	105	5.1	26	7.5
Glycine	137	6.6	49	7.5
Alanine	153	7.4	56	8.4
Cysteine[c]	22	1.1	14	2.1
Valine	139	6.7	50	7.6
Methionine	32	1.5	11	1.6
Isoleucine	102	4.9	29	4.3
Leucine	170	8.2	60	9.1
Tyrosine	71	3.4	18	2.8
Phenylalanine	103	5.0	17	2.6
Tryptophan[d]	32	1.5	2	0.2
Histidine	68	3.3	20	3.1
Lysine	185	8.9	45	6.8
Arginine	92	4.4	41	6.2
Total	2079	100.2%	661	100.1%

[a] Calculated on the basis of Ala = 153 for apoLp-I and Ala = 56 for apoLp-II. Three replicates were run; a representative analysis is shown.

[b] Includes acid + amide.

[c] Determined as cysteic acid after performic acid oxidation (Hirs, 1967).

[d] Determined directly after hydrolysis with 3 N mercaptoethanesulfonic acid (Penke *et al.*, 1974).

apoLp-II from adult lipophorin migrated with the larval apoproteins and reacted identically with antisera against larval apoproteins, again showing no cross-reactivity between apoproteins.

Exposure of Proteins in the Lipophorin Particle—In double immunodiffusion tests, antiserum against lipophorin or against apoLp-I reacted effectively against lipophorin or hemolymph (Fig. 6, *A* and *B*). However, weak precipitin lines resulted when lipophorin was reacted with antiserum against apoLp-II (Fig. 6*C*). A stronger reaction was observed in crude hemolymph. Therefore, proteins from the subnatant of the KBr density gradient were run on SDS-slab gels and blotted against anti-apoLp-II. Immunologically cross-reactive material was present at the position of apoLp-II, indicating that some of this apoprotein was free in the hemolymph.

The low immunoreactivity of lipophorin to anti-apoLp-II agreed with previous observations, indicating that the apoLp-I was more exposed to aqueous medium than apoLp-II (Pattnaik *et al.*, 1979; Mundall *et al.*, 1980).

DISCUSSION

Insect lipophorin is thought to be a lipid shuttle, reutilized many times without new synthesis or degradation (Chino and Kitazawa, 1981). In the adult locust, the lipophorin particle can absorb additional diacylglycerol from the fat body under the influence of the decapeptide adipokinetic hormone (Mwangi and Goldsworthy, 1981). Lipophorin thereupon increases in size and decreases in density to the low density lipoprotein class. These changes are reversed as diacylglycerol is deposited for use as fuel in the flight muscles. Thus, unlike mammalian lipoproteins, which may change in density but not fluctuate, insect lipophorin fluctuates between lipid density classes in the course of its transport function. Diacylglycerol transport is apparently facilitated by association with a small protein, designated C protein (Mwangi and Goldsworthy, 1981; van der Horst *et al.*, 1981; Wheeler and Goldsworthy 1983a, 1983b), though the protein and nature of its association with lipophorin have not been characterized. While not present in larvae, a similar protein has recently been isolated from adult M. sexta.[4] We propose to call this small apoprotein apoLp-III, in accordance with our proposed terminology and to avoid premature comparison with mammalian apolipoproteins C.

In order to understand how insect lipophorin functions during lipid transport, we need to know more about the structure of the particle and of its apoprotein components. We have developed a gentle and rapid density gradient procedure for isolating the larval lipophorin from the hemolymph of *M. sexta* and an efficient gel permeation chromatography procedure for separating the apoproteins. The larval lipophorin is homogeneous with respect to molecular weight and density, while lipophorin from adult *M. sexta* is polydisperse and less dense than the larval form and thus resembles the diacylglycerol-loaded lipophorin of the adult locust (Shapiro and Law, 1983).

We have substantially improved separation of the apoproteins by gel permeation chromatography through use of guanidine hydrochloride as a chaotrope rather than SDS, which was used previously (Pattnaik *et al.*, 1979). We performed complete amino acid analysis on the isolated apoproteins and found that the cysteine and tryptophan contents, not previously determined, were of particular interest. ApoLp-I contained 29–32 residues/mol (1.5 mol %) of tryptophan, compared to only 2 residues/mol (0.2 mol %) for apoLp-II. This observation accounts for the low absorbance of apoLp-II at 280 nm seen during chromatography in 6 M guanidine hydrochloride (Fig. 3). Cysteine content of the apoproteins also differed; apoLp-I contained 22 residues/mol (1.1 mol %), while apoLp-II contained 14 residues/mol (2.1 mol %). Assuming that most cysteine residues are involved in disulfide linkages, the higher percentage of cysteine in apoLp-II may contribute to a more compact structure.

Some studies on the lipophorin of *Locusta migratoria* have demonstrated a single apoprotein that is comparable in molecular weight to apoLp-II of *Manduca* lipophorin (Gellissen and Emmerich, 1980; Gellissen and Wyatt, 1981), while others have found both apoproteins corresponding to those of *M. sexta* (Chino *et al.*, 1981b; Chino and Kitazawa, 1981). Chino and Kitazawa (1981) found a definite difference in the apoproteins of locust lipophorin; apoLp-I stained for carbohydrate in SDS-polyacrylamide gels, while apoLp-II did not. We have isolated two distinct apoproteins from *Manduca* lipophorin, both of which are stained by the periodate-Schiff reagent and both of which bind fluorescein labeled concanavalin A, indicating that they are both high mannose glycoproteins. We have clearly demonstrated through immunological methods that they are not homologous (Fig. 4). This effectively rules out aggregation of apoLp-II as the origin of apoLp-I and indicates that apoLp-II is not a result of fragmentation of genes for apoLp-I or a processing product of an apoLp-I transcript. It is possible, however, that the two apoproteins are discrete products of a single large gene or messenger, as are the apoproteins of some vitellogenins (Harnish *et al.*, 1982). We also showed that two apoproteins found in adult lipophorin are immunologically identical to those in larval lipophorin (Fig. 5).

We propose that the apoproteins serve two general roles, maintenance of particulate structure and facilitation of lipid transfer. In mammalian systems, lipoprotein structure is maintained by a combination of hydrophilic surface interac-

[4] Kawooya, J. K., Keim, P. S., Ryan, R. O., Samaraweera, P., Shapiro, J. P., and Law, J. H., submitted for publication.

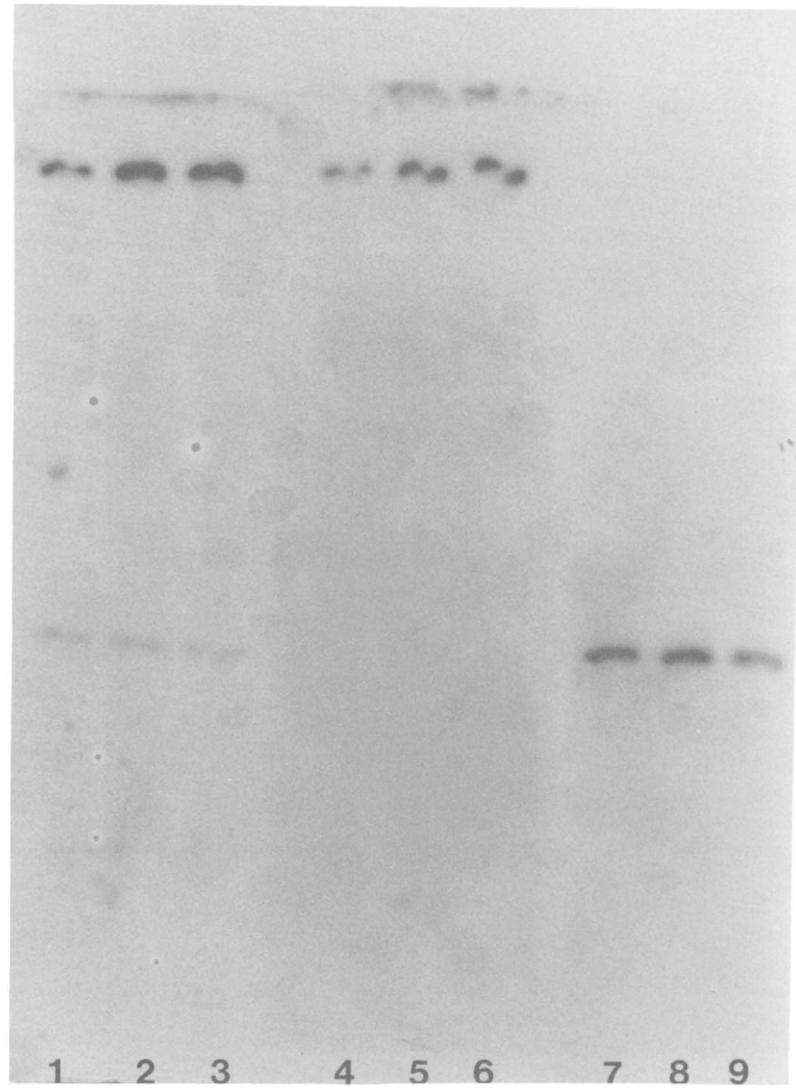

FIG. 5. **Cross-reactivity of larval and adult apoproteins to antisera against larval apoproteins and lipophorin.** Lipophorin (*lanes 1*, *4*, and *7*) or hemolymph (*lanes 2*, *5*, and *8*) from larvae or lipophorin from adults (*lanes 3*, *6*, and *9*) was applied to a 5% SDS-polyacrylamide gel, electrophoresed, transferred electrophoretically to nitrocellulose, and reacted with anti-lipophorin (*lanes 1–3*), anti-apoLp-I (*lanes 4–6*), or anti-apoLp-II (*lanes 7–9*) as above.

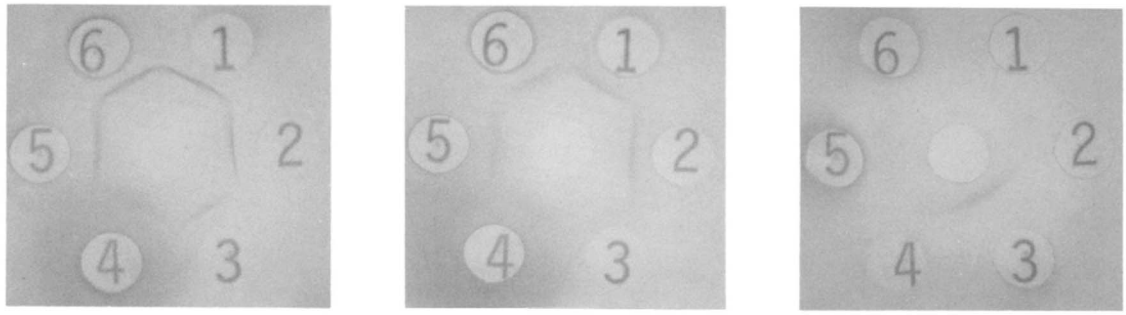

FIG. 6. **Double immunodiffusion of lipophorin and hemolymph against antisera to apoproteins or whole lipophorin.** 10 μl of antiserum against (*A*) whole lipophorin (5-fold dilution), (*B*) apoLp-I (2-fold dilution), or (*C*) apoLp-II (undiluted) was placed into *center wells*. Peripheral wells in *A* and *B* contained 10 μl of: lipophorin (10 μg, *well 1*; 2 μg, *well 2*; 1 μg, *well 3*), whole hemolymph (2-fold dilution, *well 4*; 5-fold dilution, *well 5*; 10-fold dilution, *well 6*). Peripheral wells in *C* contained 10 μl of: apoLp-II in PBS (>10 μg, *well 1*; >5 μg, *well 2*), whole hemolymph (2-fold dilution, *well 3*), or lipophorin (20 μg, *well 4*; 10 μg, *well 5*; 5 μg, *well 6*). Samples were allowed to diffuse for 3 days at room temperature and 3 days at 4 °C. Plates were dried and stained as described under "Experimental Procedures."

tions and hydrophobic core interactions (Shen *et al.*, 1977). The former are contributed by polar apoprotein residues and lipid groups, the latter by apolar lipids. The small proportion of apolar hydrocarbons, triacylglycerols, and sterol esters in lipophorin may demand a greater role for apoproteins in formation of an apolar core. This concept is supported by our experiments on relative accessibility of the two apoproteins to proteases, immunoglobulins, and radiolabeling reagents.

An earlier report found apoLp-I to be much more susceptible to trypsin digestion than apoLp-II (Pattnaik *et al.*, 1979) and radioiodination (Mundall *et al.*, 1980). Here we have demonstrated that apoLp-I in the intact particle is more susceptible than apoLp-II to reaction with antibodies. Low accessibility of apoLp-II to bulky probes suggests that this polypeptide is sheltered from the aqueous environment and may lie partly within the particle, perhaps constituting a part of the core.

Though lipophorins readily exchange lipid (diacylglycerol and cholesterol) with tissue both *in vitro* (Chino and Gilbert, 1965; 1971) and *in vivo* (van der Horst *et al.*, 1981), no exchange mechanism has yet been proposed (Chino and Kitazawa, 1981). However, the apoproteins, especially apoLp-I, may participate in exchange by reversible association with plasma membranes. In mammalian systems, polar lipids exchange from lipoproteins more readily than the apolar triacylglycerols and cholesterol esters found in the lipoprotein core (Morton and Zilversmit, 1982). Several soluble plasma proteins that facilitate triacylglycerol and cholesteryl ester exchange have been isolated (Rajaram *et al.*, 1980; Zilversmit *et al.*, 1975; Pattnaik *et al.*, 1978; Ihm *et al.*, 1980, 1982; Chajek and Fielding, 1978). Since insect lipophorins contain a larger proportion of polar lipid than mammalian lipoproteins, a larger proportion of lipid should be freely exchangeable. This does not exclude the presence of hemolymph proteins that facilitate lipid transfer, especially in life stages requiring rapid turnover of polar lipid. In adult *M. sexta* a new apoprotein, apoLp-III, is found in the hemolymph. ApoLp-III, which can reversibly associate with lipophorin, may serve a lipid transfer function during the adult stage, a period of great demand for lipid, both as flight fuel in many insects (Bailey, 1975) and as a constituent of egg yolk. Furthermore, apoLp-III might serve as a recognition signal to promote interaction between lipophorin and muscle cell membranes, or it might serve to activate lipolytic enzymes at the muscle to aid in assimilation of transported diacylglycerol, much as apolipoprotein C-II in human very low density lipoprotein serves to activate lipoprotein lipase in peripheral tissue (Smith *et al.*, 1978).

Acknowledgments—We wish to thank Dr. R. O. Ryan, Dr. J. Kawooya, and E. Osir for critical review of the manuscript, M. Gonzales for technical assistance, and to express appreciation to Dr. J. P. Reinecke, United States Department of Agriculture, for providing experimental animals.

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Supplement to: Structural Studies on Lipophorin, An Insect Lipoprotein

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EXPERIMENTAL PROCEDURES

Animals--Manduca sexta (tobacco hornworm) eggs were obtained from the U. S. Department of Agriculture, Fargo, North Dakota. Eggs were hatched and larvae reared as previously described (Kramer et al., 1974).

Lipophorin Preparation--Lipophorin was isolated from whole larval hemolymph by single spin density gradient ultracentrifugation in a Beckman VTi50 vertical rotor, using a procedure modified from Chung et al. (1981). Fifth instar larvae were bled through prolegs into phosphate buffered saline (PBS; 0.15 M NaCl/0.10 M sodium phosphate/0.05 percent EDTA pH 7.0) containing phenylthiourea or glutathione (5 mM) to inhibit melanization. Approximately 0.5 ml hemolymph were collected per larva, to total 20-40 ml. Hemocytes were sedimented by centrifugation for 15 min at 5000g (4°C), the supernatant collected, and KBr and PBS were added with stirring to a final concentration of 44.3 g KBr/100 ml of solution. The KBr/hemolymph mixture (density = 1.31) was placed into Beckman 39 ml QuickSeal tubes, 19.5 ml/tube, and overlayered with 19.5 ml of fresh 0.9 percent NaCl (density = 1.007). Tubes were placed into a Beckman VTi50 vertical rotor and centrifuged at 50,000 rpm for 4 hr at 10°C in a Beckman model L8-70R centrifuge with slow acceleration feature. A tube lacking hemolymph was centrifuged with sample tubes, fractionated, the refractive index read, and densities calculated from the refractive index of KBr at 25°C.

Under these conditions the gradient ranged from 1.03 to 1.30 g/ml in density (Fig. 1). Lipophorin, which appeared as a single bright yellow band in the center of the tube, separated from other sedimenting proteins by several centimeters, as indicated by the bright blue color of the biliverdin-containing protein, insecticyanin (Cherbas, 1973), in the lower third of the tube. As demonstrated by repeated centrifugation of isolated lipophorin (Fig. 1) and by SDS-polyacrylamide electrophoresis of gradient fractions (Fig. 2), lipophorin was located at a density of 1.13 ± 0.05 g/ml.

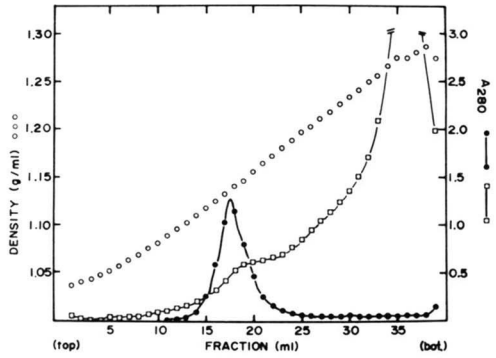

Fig. 1. Density gradient ultracentrifugation of hemolymph proteins and isolated lipophorin. Hemolymph was collected and centrifuged in a density gradient as described, along with a tube containing previously isolated lipophorin and a blank tube containing only salt solutions. Each tube was fractionated from the top, and the absorbance at 280 nm (tubes with hemolymph or lipophorin) or refractive index (blank tube) read from 1 ml fractions. o---o, lipophorin absorbance; ---, total absorbance from crude hemolymph.

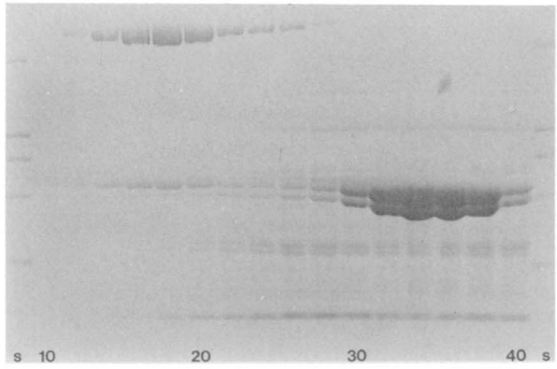

Fig. 2. SDS-polyacrylamide gel electrophoresis of density gradient fractions. Hemolymph was collected and centrifuged as described, and 2 ml fractions collected from the bottom of the density gradient tube. Fractions were dialyzed overnight against PBS in a microdialyzer (BRL) and applied to a 3-8 percent SDS-polyacrylamide gradient slab gel. Numbers indicate mls from top of the tube; mls 1-8 were omitted, since they contained no significant amounts of protein. Standards (S): myosin (200,000), B-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), and ovalbumin (45,000).

After centrifugation, the yellow lipophorin was collected, 4.8-5.3 cm from the bottom of the tube, by puncturing the side of the tube and withdrawing 3-4 ml/tube, or by puncturing the top or bottom of the tube and collecting the entire volume in 1 ml fractions for density gradient and protein content analysis. Lipophorin collected through the side of the tube was dialyzed 3-4 days against 4 changes of PBS and stored for up to 3 weeks in capped tubes at 4°C under nitrogen. Fractions for application to SDS polyacrylamide gels were collected from the top of the tube and individually dialyzed against PBS prior to electrophoresis.

Isolation of Apoproteins from Purified Lipophorin--Apoproteins were dissociated in 6M guanidine HCl (U.S. Biochemicals) and isolated on a Sepharose 6B-CL column equilibrated in 6M guanidine HCl buffer. Guanidine HCl (1.72 g) was added to lipophorin (1.8 ml of 1-2 mg/ml in PBS) and gently mixed to give 3.0 ml of a 6M solution. The solution was then heated to 30°C for 1 h with occasional gentle mixing, cooled, and applied to a 69 x 1.0 cm column of Sepharose 6B-CL equilibrated in 50 mM phosphate/6M guanidine HCl/pH 7.0 (guanidine HCl buffer). The column was eluted at 8 ml/h, and 2 ml fractions collected. This procedure was modified for preparative chromatography by adding 22.9 g of guanidine HCl to 24.0 ml of lipophorin to give 40.0 ml, applying to a 90 x 2.5 cm column of Sepharose 6B in guanidine buffer, and eluting at 60 ml/h. Fractions of 5 ml were collected, and peak fractions showing absorance at 280 nm were pooled and concentrated to 10 percent (apoLp-II) or 25 percent (apoLp-I) of original volume in an Amicon ultrafiltration device with YM-30 membrane, then dialyzed 2-3 days vs 3 or 4 changes of distilled deionized water and lyophilized or stored as precipitated protein at 4°C in water with 0.04 percent sodium azide.

The apoproteins readily dissociated when lipophorin was dissolved in 6M guanidine HCl. Earlier apoprotein separations were done in SDS (Pattnaik et al., 1979), but this procedure yielded an impure apoLp-II. Dissociation in CHAPS (a zwitterionic steroid detergent), urea, or after extraction of lipids in organic solvents also yielded unsatisfactory results: treatment with either 8M urea and/or organic solvents caused precipitation of the lipoprotein. In contrast, 6M guanidine HCl readily and completely dissociated the apoproteins, allowing effective separation on a gel filtration column in 6M guanidine HCl (Fig. 3). SDS-polyacrylamide gel electrophoresis demonstrated that apoLp-I and apoLp-II were effectively separated (Fig. 4). Recoveries of apoLp-I and apoLp-II were 58 percent and 29 percent, respectively. Recoveries especially of apoLp-I, could be increased by omitting the concentration step (Table I).

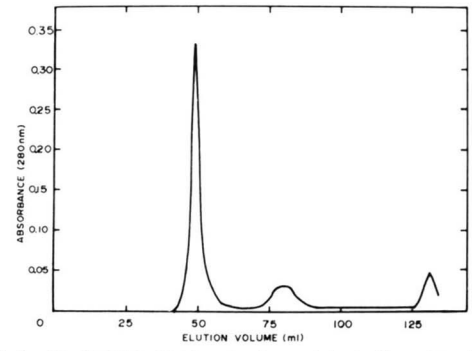

Fig. 3. Gel permeation chromatography of apoproteins in 6M guanidine HCl. Lipophorin was dissociated in guanidine HCl and chromatographed in guanidine HCl/phosphate buffer (pH 7.0) on a preparative gel permeation column, as described. Absorbance at 280 nm was monitored with a Gilson Holochrome monitor and a Linear strip chart recorder.

Purification Step	A_{280}	Volume (ml)	A_{280} x Volume	Equivalent wt. (mg)	Percent Recovery
Guanidine HCl					
Lipophorin	1.177	24.0	40.7	65.6	--
ApoLp-I	--	--	--	50.9	--
ApoLp-II	--	--	--	14.7	--
Column Eluent					
ApoLp-I	0.623	59.0	36.8	51.5	100
ApoLp-II	0.26	62.5	1.63	5.4	37
Concentrated					
ApoLp-I	1.226	25.7	31.5	44.1	87
ApoLp-II	0.171	10.0	1.71	5.7	39
Lyophilized					
ApoLp-I	--	--	--	29.7	58
ApoLp-II	--	--	--	4.3	29

Table I. Recovery of apoproteins following gel permeation chromatography in guanidine HCl. Lipophorin was dissociated in guanidine HCl and chromatographed on a preparative gel permeation column as described. Expected yield of each apoprotein was calculated from A_{280} of the undissociated lipophorin and specific absorbance of each apoprotein. Recovery at each step was calculated from A_{280} and specific absorbances in guanidine HCl, or from weight of lyophilized apoproteins.

Immunology--Intact lipophorin (0.7 mg) in PBS, or isolated apoLp-I (0.6 mg) or apoLp-II (0.8 mg) in 0.5 ml distilled water, was emulsified in 1.5 ml Freund's complete adjuvant. Emulsions were injected intramuscularly into all four limbs of New Zealand white rabbits, 0.5 ml/limb. After five to nine weeks, animals were boosted intramuscularly in the hind limbs with 0.5 mg of whole lipophorin, 0.5 mg of apoLp-I, or 0.2 mg of apoLp-II emulsified in 0.75 ml Freund's incomplete adjuvant (Miles). Blood was collected by heart puncture one to three weeks later, and sera separated from clots after three to six hours at room temperature, then freed of cells by centrifugation at 10,000g.

Double radial immunodiffusion (Ouchterlony, 1968) was performed in 1.8 mm thick 1 percent agarose (SeaKem, LE grade) in PBS on 95 x 45 mm plates (Miles), stained with Coomassie brilliant blue R in 50 percent methanol/9 percent acetic acid and destained in 50 percent methanol/9 percent acetic acid.

Electrophoresis--SDS-polyacrylamide slab gel electrophoresis was performed by the method of Laemmli (1970) on 0.15 x 18 x 12 cm acrylamide separating gels with 1 cm deep stacking gels. Gradient gels were formed with a gradient maker (BRL). Coomassie stained gels were scanned with a Schoeffel SDC3000 scanning spectrodensitometer and peaks directly quantitated with a Hewlett-Packard 3390A integrator.

Preparative gel electrophoresis utilized the same electrophoretic system on a BRL preparative apparatus. Separating gels of 3 cm and stacking gels of 0.4 cm were used, and gels were run at approximately 5.5 mA and less than 1 watt power. Fractions were eluted with SDS reservoir buffer by peristaltic pump and collected in 1 ml fractions. Pooled fractions were dialyzed overnight against 0.1 percent SDS at 22°C and assayed in SDS by the Folin-phenol protein assay of Peterson (1983). Standards of purified, lyophilized ApoLp-I and ApoLp-II were boiled in 1 percent SDS for 0.5 hr and diluted to 0.1 percent SDS and 0.1 mg/ml prior to use. ApoLp-I showed 0.57 times the slope of ApoLp-II in this assay.

For immunological analysis, proteins in SDS gels were electrophoretically transferred to nitrocellulose (Millipore HAWP, 45 μM), reacted with specific antisera (Towbin et al., 1979), and then with Staphlococcus protein A (Sigma) (Burnette, 1981) which had been labeled by reaction (15 min, 25°C) with Na-[^{125}I] (NEN) and Iodobeads (Pierce; Markwell, 1982). Protein A-bound immunoglobulins were visualized by exposing Kodak X-Omat AR X-ray film to nitrocellulose blots at -70°C for 8-24 h in a Dupont cassette with Lightning Plus Intensifier Screen.

Slab gels were stained for carbohydrate using two different procedures. For paraldehyde-Schiff staining, the method of Kapitany and Zebrowski (1973) was followed. Gels were stained with fluorescein isothiocyanate conjugated-concanavalin A (Miles) by the method of Furlan et al. (1979).

Amino Acid Analysis--Cysteine and cystine residues were oxidized to cysteic acid by the method of Hirs (1967), hydrolyzed 24 h in vacuo at 110°C in 6N HCl, and analyzed as below. Tryptophan was estimated spectrophotometrically (Edelhoch, 1967) and by amino acid analysis preceded by mild hydrolysis in 3N mercaptoethanesulfonic acid (22 h, 110°C) (Penke et al., 1974). Analyses were performed on a Dionex D-300 amino acid analyzer using the standard column and three-buffer system suggested by the manufacturer, with an additional sodium citrate buffer (0.35 N Na^+/pH 5.26) added for tryptophan elution after the B buffer. Peaks were directly integrated on a Hewlett-Packard 3388A integrator.

High Performance Liquid Chromatography (HPLC)--Gel permeation HPLC was performed on a Varian 5000 chromatograph with UV-50 detector using a Bio-Rad TSK-400 column in 0.15 M Na_2SO_4/0.02 M Na_2PO_4/0.02 percent NaN_3/pH 6.8.

Spectrophotometry--UV and visible spectrophotometry was performed on a Perkin-Elmer Lambda 3 spectrophotometer.