Apolipophorin III in locusts: purification and characterization

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Abstract Three molecular species of apolipophorin III were purified from adult locust hemolymph by gel filtration and ion-exchange chromatography, and named apo-III-a, apo-III-b, and apo-III-c, respectively. They were indistinguishable by SDS-polyacrylamide gel electrophoresis, immunodiffusion, and in amino acid composition; however, they had different isoelectric points (5.43 for a, 5.11 for b, and 4.98 for c) and, therefore, could be separated by native- or urea-gel electrophoresis. All three apo-IIIs were glycoproteins and contained fucose, mannose, and glucosamine. The total sugar content amounted to about 11% for each of the three apo-IIIs. The molecular weight of apo-III determined by SDS-polyacrylamide gel electrophoresis was approximately 20,000, almost equivalent to the native molecular weight (approximately 19,000) estimated by the sedimentation-equilibrium method. This indicated that the locust apo-III exists in hemolymph as a monomeric form. It was demonstrated that a total 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 locust adipokinetic hormone. —**Chino, H., and M. Yazawa.** Apolipophorin III in locusts: purification and characterization. *J. Lipid Res.* 1986. **27:** 377–385.

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

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 (2–5). Lipophorin generally comprises two apoproteins, a heavy chain (apo-I) with a molecular weight of 250,000 and a light chain (apo-II) with a molecular weight of 80,000–85,000 (2, 6, 7).

Insect adipokinetic hormone (AKH), first discovered in locusts by Mayer and Candy (8), is released from the corpora cardiaca during flight and stimulates the loading by lipophorin of diacylglycerol from the fat body, resulting in the formation of larger lipophorin particles with increased diacylglycerol content (9–11). Injection of AKH into adult locusts causes the association of a low molecular weight, non-lipid-containing protein with lipophorin (12–15). Similar observations have been extended to the adult tobacco hornworm, *Manduca sexta*, by Shapiro and Law (16) who proposed the term apolipophorin III (apo-III) for the low molecular weight protein that becomes associated with lipophorin in response to AKH injection. The purification of apo-III from the hemolymph of *M. sexta* has been recently achieved in their laboratory (17).

Our previous report (18) extended these observations to the locust, in which we demonstrated that the injection of AKH promotes the association of free apo-III in hemolymph with lipophorin and the loading of diacylglycerol from the fat body by lipophorin to produce larger, lower density lipophorin particles. The structural changes of lipophorin in response to AKH are completely reversible, so that within 24 hr of AKH injection, the apo-III dissociates from lipophorin and the size and density of lipophorin return to the original values observed in resting locusts (18).

The effect of AKH in locust has been demonstrated in vitro using partially purified lipophorin and crude C_2-protein (equivalent to apo-III) by Van Heusden, Van Der Horst, and Beenakkers (19). However, the mechanism by which the above structural changes of lipophorin are induced under the action of AKH still remains unresolved. The resolution of this mechanism requires the availability of purified apo-III. The present study describes the purification and characterization of locust apo-III. Evidence is also presented to indicate that locust apo-III largely differs from *M. sexta* apo-III in physico-chemical properties.

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

Abbreviations: apo-III, apolipophorin III; AKH, adipokinetic hormone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

AKH-injected locusts (90 min after injection) by the "flushing out" method (20).

Chemicals

Locust adipokinetic hormone (21), a blocked decapeptide, was purchased from Peninsula Laboratories. Standard proteins of known molecular weight were obtained from Boehringer, Mannheim. Ultrogel AcA 44 and DEAE-Toyopearl 650M were obtained from LKB, Sweden and Toyo-Soda Co., Japan, respectively. All other chemicals were of analytical grade. Glass-redistilled water was used throughout the experiments.

Preparation of activated lipophorin

The activated lipophorin associated with apo-III was prepared by potassium bromide density-gradient ultracentrifugation from the hemolymph of male locusts following injection of AKH (10 pmol/insect). This method was first employed by Shapiro and Law (16) for *M. sexta*. The detailed procedure is described in our previous report (18). After centrifugation at 50,000 rpm for 4 hr, the lipophorin fraction appeared as a single distinct yellow band, and the darkest segment (0.6–0.7 ml) of the yellow band was collected directly by a Pasteur pipet and used as a reference for apo-III on SDS-PAGE. The lipophorin-free subnatant fraction obtained after the above centrifugation was used in some experiments.

Polyacrylamide gel electrophoresis

Native-PAGE was run on 10% gels and the gels were stained with Coomassie blue G-250 by a rapid staining method (22). SDS-PAGE was performed using 10% gels according to the method of Weber and Osborn (23) and the gels were stained with Coomassie blue. Urea-PAGE was run according to the method of Perrie and Perry (24). The sample was treated with 8 M urea and applied to a 5% gel containing 8 M urea; electrophoresis was at pH 8.9 with 20 mM Tris-glycine buffer. The lipophorin prepared by density gradient ultracentrifugation was dialyzed against 10 mM phosphate buffer (pH 6.9) to remove excess potassium bromide before treatment with SDS or urea.

The isoelectric point of purified apo-III was determined by isoelectric focusing on 5% polyacrylamide gel (2 × 100 mm cylinders) with 2% Pharmalyte (pH 3–10.5). Ethylenediamine (10 mM) and iminodiacetic acid (10 mM) were used as the cathode and anode solutions, respectively. Other procedures followed the instruction book from Pharmacia Fine Chemicals (25).

Molecular weight determination

The native molecular weight of purified apo-III (dissolved in 0.1 M NaCl) was determined by the rapid sedimentation-equilibrium method of Yphantis (26), in a Hitachi analytical ultracentrifuge (model 282) equipped with a photoelectric scanning system and data analyzer (model DA-7). The equilibrium was established at 14,000 rpm after 6 hr run at 15°C.

Preparation of antibody and immunodiffusion test

The purified apo-III (about 1 mg) emulsified with Freund's complete adjuvant was injected subcutaneously on the back of a rabbit. A booster injection was made 2 weeks after the initial injection. Two weeks later, the rabbit was bled and IgG was separated from serum by ammonium sulfate precipitation at 40% saturation. Following dialysis against Tris-buffered saline (20 mM Tris-HCl containing 150 mM NaCl, pH 7.5), the anti-apo-III IgG thus obtained was stored frozen at −80°C. Immunodiffusion tests were carried out after the method of Ouchterlony (27) using 1% agarose gel containing Tris-HCl saline. After antigens and antibody were allowed to diffuse at 5°C for 18 hr, the gel plates were examined for precipitin lines.

Determination of amino acid, amino sugar, and neutral sugar

The amino acid composition of apo-III was determined on an automatic amino acid analyzer (Hitachi, model 835) after hydrolysis with 6 N HCl at 110°C for 24 hr. For determination of tryptophan, the apo-III was hydrolyzed with 4 N methanesulfonic acid at 115°C for 24 hr (28). The qualitative analysis of amino sugar covalently associated with apo-III was performed on the same amino acid analyzer after hydrolysis with 3 N HCl at 100°C for 16 hr. The quantitative determination of the amino sugar was performed colorimetrically after the method developed by Smith and Gilkerson (29), using glucosamine as standard.

The neutral sugar composition of apo-III was analyzed by gas–liquid chromatography. The purified apo-III was first methanolyzed with 0.5 N HCl in methanol (total volume 0.5 ml) at 80°C for 20 hr. After methanolysis, silver carbonate was added to remove chloride and, following the addition of 0.1 ml of anhydrous acetic acid, the solution was allowed to stand for 6 hr at room temperature. After centrifugation, the supernatant was dried completely under a stream of nitrogen and the remaining methyl glycosides were then trimethylsilylated (TMS). An aliquot of the TMS-derivatives was analyzed by gas–liquid chromatography (Shimadzu, GC-4CM) equipped with a data analyzer (Chromatopac E-1A) using a column of 1.5% OV-1 on Chromosorb W (column size, 2 m × 3 mm) with N_2 as carrier gas at a constant temperature of 180°C. For quantitative determination, mannitol was used as an internal standard. The TMS-derivatives were analyzed by mass spectrometry on a mass spectrometer (JOEL, JMS-D300) for further identification.

Protein determination

The amount of protein was determined by the Biuret method or the method of Lowry et al. (30), using bovine serum albumin as standard.

RESULTS

Purification of locust apolipophorin III

All the purification procedures were carried out at 0°–3°C. Lipophorin and vitellogenin (female-specific protein) were first removed from freshly collected pooled hemolymph of resting locusts (usually about 100 locusts of both sexes for each run of purification) by specific precipitation under low ionic concentration, as developed in this laboratory for the purification of both lipophorin and vitellogenin (2, 31). After centrifugation at 10,000 *g* for 10 min to remove precipitates, the lipophorin- and vitellogenin-free supernatant (about 300 ml or more) was stored frozen at −80°C until use. In some experiments, the supernatant was used immediately without freezing.

As the first step of purification, 20 ml of a solution containing 50 mM EDTA and 2 mM phenylmethanesulfonyl fluoride was added to 300 ml of the supernatant to prevent possible proteinase action. The supernatant was then brought to 40% saturation by adding solid ammonium sulfate and allowed to stand for 2 hr. After removing any precipitate by centrifugation at 12,000 *g* for 20 min, the supernatant was brought to 90% saturation by further addition of ammonium sulfate and allowed to stand for 4 hr. The precipitate collected by centrifugation at 12,000 *g* for 20 min was dissolved with a small volume (2–3 ml) of 0.1 M NaCl buffered with 10 mM Tris-HCl, pH 8.0, and chromatographed on Ultrogel AcA 44 (2.7 × 90 cm) equilibrated with the same NaCl solution. The column was eluted with the above solution at a flow rate of 28 ml/hr, and 5-ml aliquots were collected.

The elution profile is illustrated in **Fig. 1** and demonstrates four major peaks, identified as peak I, peak II, peak III, and peak IV. Each fraction was tested for the presence of apo-III on SDS-PAGE using activated lipophorin as a reference. The results indicate that apo-III appears principally in peak III and in some fractions of peak IV, but no apo-III is detected in the other fractions (data of SDS-PAGE are not shown but details will be described later). The peak III fractions (shadowed) were combined and dialyzed against 60 mM ammonium bicarbonate overnight. The dialyzed solution was subsequently subjected to ion-exchange chromatography on DEAE-Toyopearl 650M (2.2 × 25 cm) equilibrated with 60 mM ammonium bicarbonate. The column was first eluted with the same solution at a flow rate of 30 ml/hr for 6 hr, and then eluted with a linear concentration gradient of

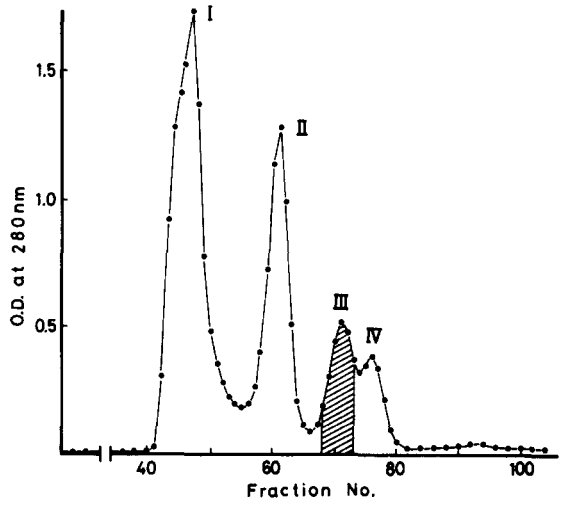

Fig. 1. Elution profile of the lipophorin and vitellogenin-free supernatant on Ultrogel AcA 44. Five ml was collected in each tube. The peak-III fraction (shadowed) was subjected subsequently to ion-exchange chromatography.

ammonium bicarbonate from 60–170 mM at the same flow rate, and 10-ml aliquots were collected.

The elution profile is illustrated in **Fig. 2** and demonstrates three distinct peaks that are designated as apo-III-a, apo-III-b, and apo-III-c, respectively. These three fractions were dialyzed several times against large volumes of distilled water and then lyophilized. The lyophilized samples were tested for apo-III on SDS-PAGE. The results are shown in **Fig. 3** and clearly reveal that both apo-III-b and apo-III-c are homogeneous and contain no other proteins (Fig. 3, D, E). However, the apo-III-a fraction is not homogeneous and is contaminated with a lower molecular weight protein (Fig. 3, B). The crude apo-III-a fraction was rechromatographed on Ultrogel AcA 44. The elution

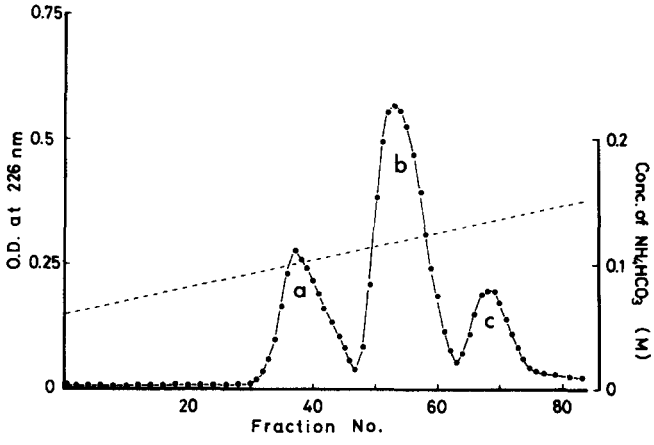

Fig. 2. Elution profiles of the peak-III fraction by a linear concentration gradient of ammonium bicarbonate (60–170 mM) on DEAE-Toyopearl 650M. Ten ml was collected in each tube.

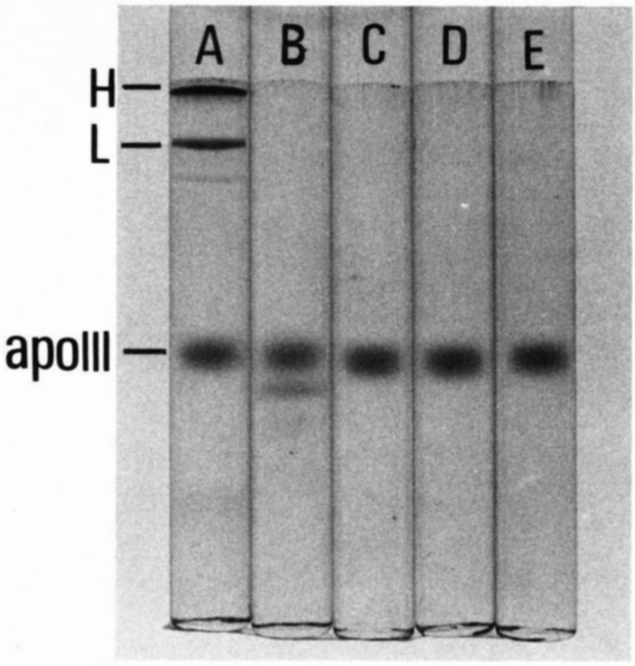

Fig. 3. SDS-PAGE (10% gel) of three apo-IIIs. A, Activated lipophorin as reference (15 μg of protein); B, crude apo-III-a (5 μg); C, rechromatographed apo-III-a (5 μg); D, apo-III-b (5 μg); E, apo-III-c (5 μg). H, heavy chain; L, light chain.

profile indicates a major peak followed by a shoulder which probably represents the contaminating protein (**Fig. 4, A**). The major peak fractions (shadowed) were combined, dialyzed several times against distilled water, lyophilized, and tested for homogeneity on SDS-PAGE. As shown in Fig. 3 C, the rechromatographed apo-III-a is sufficiently pure and the minor contaminant is completely eliminated. Fig. 3 also demonstrates that the three apo-IIIs are not distinguishable on SDS-PAGE.

The homogeneity of apo-III-a (rechromatographed), apo-III-b, and apo-III-c was also demonstrated by immunodiffusion in which IgG prepared against apo-III-b was used as an antibody. As shown in **Fig. 5**, both apo-III-a and apo-III-c cross-react with anti-apo-III-b and a distinct, single precipitin line is exhibited, indicating that the three apo-IIIs are not immunologically distinguishable.

17K protein as a contaminant

A preliminary experiment indicated that the contaminating protein found in the crude apo-III-a fraction on SDS-PAGE is indistinguishable from apo-III-a on native-PAGE; thus it was desirable to isolate the contaminating protein. The close association of peak III and peak IV is indicated in Fig. 1 and suggests that the contaminating protein may be derived from the protein of peak IV. Therefore, the peak IV fractions were combined and subjected to rechromatography on Ultrogel AcA 44. The elution profile

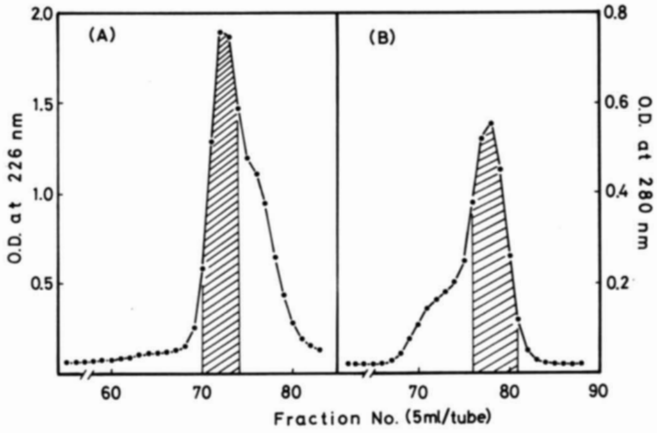

Fig. 4. Rechromatography on Ultrogel AcA 44 of the crude apo-III-a (A) and the peak-IV fraction (B). The lyophilized crude apo-III-a (pooled from three runs of the purification) was dissolved with a small volume of 0.1 M ammonium bicarbonate and applied to the column (2.7 × 90 cm) which was eluted with 0.1 M ammonium bicarbonate at a flow rate of 28 ml/hr. The peak-IV fractions (see Fig. 1) were combined, dialyzed against distilled water, and lyophilized. The lyophilized sample (pooled from two runs of the purification) was rechromatographed as above.

is illustrated in Fig. 4 B and demonstrates a profile that is almost opposite to that of crude apo-III-a (Fig. 4, A); a shoulder is followed by the major peak. The shoulder fraction was shown to be primarily apo-III-a by SDS-PAGE (data not shown). The major peak fractions (shadowed) were combined, dialyzed aginst distilled water, lyophilized, and tested for homogeneity on SDS-PAGE. As illustrated in **Fig. 6 (A, B, C)**, the rechromatographed preparation

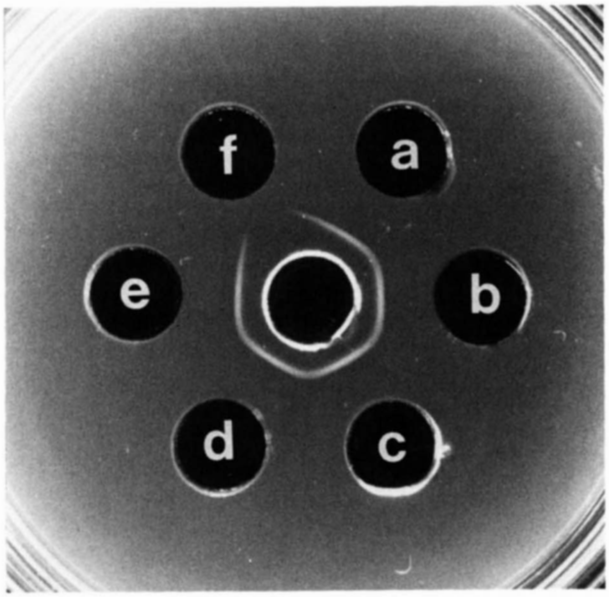

Fig. 5. Ouchterlony's double diffusion test. Center well contained 300 μg of anti-apo-III-b IgG. a, Male locust hemolymph (30 μg of protein); b, apo-III-a (10 μg); c, apo-III-b (10 μg); d, apo-III-c (10 μg); e, activated lipophorin (20 μg); f, apo-III-free resting lipophorin (20 μg).

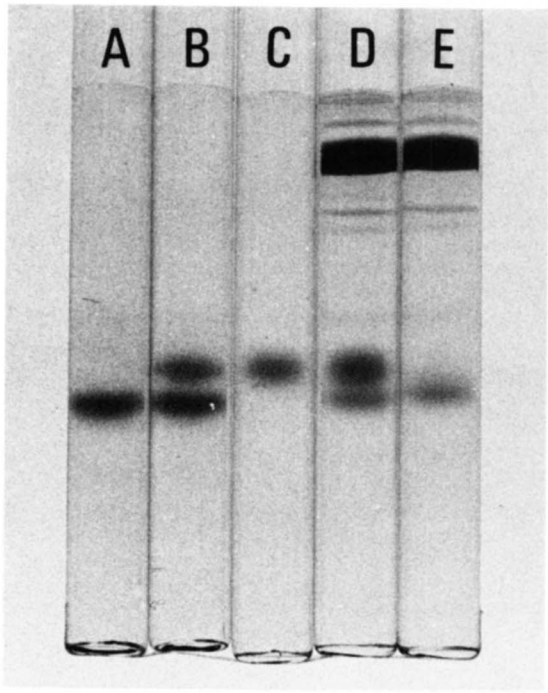

Fig. 6. SDS-PAGE (10% gel) of 17K protein, apo-III-a, and the lipophorin-free subnatant fraction obtained after density gradient centrifugation of resting or AKH-treated hemolymph. A, 17K protein (5 μg); B, mixture of 17K protein and apo-III-a (5 μg of each); C, apo-III-a (5 μg); D, lipophorin-free subnatant fraction from resting hemolymph (20 μg); E, lipophorin-free subnatant fraction from AKH-treated hemolymph (20 μg).

migrates as a distinct single band and migrates faster than apo-III-a. The molecular weight of the newly purified protein was determined on SDS-PAGE with activated lipophorin and several standard proteins. The molecular weight was estimated to be approximately 17,000 (**Fig. 7**) and, thus, the protein is defined as the "17K" protein in this report. The estimated molecular weight of apo-III

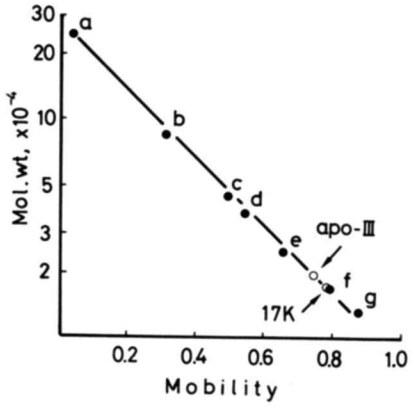

Fig. 7. Relationship of the mobilities on SDS-PAGE and the molecular weights. a, Lipophorin heavy chain (250,000); b, lipophorin light chain (85,000); c, egg albumin (45,000); d, alcohol dehydrogenase (37,000); e, chymotrypsinogen (25,000); f, myoglobin (17,200); g, cytochrome c (12,500).

itself was about 20,000, confirming the value proposed in the previous study (18). The 17K protein does not associate with lipophorin in response to AKH and remains free in hemolymph after the injection of AKH (Fig. 6, D, E) (see also Fig. 3, A).

Electrophoretic behavior of three apo-IIIs on native- or urea-PAGE and their isoelectric points

The data presented in the previous section indicate that the three apo-IIIs are indistinguishable on the basis of their mobility on SDS-PAGE (Fig. 3). However, as illustrated in **Fig. 8 (C, D, E)**, the three apo-IIIs are separable on native-PAGE; they migrate rapidly in the order of apo-III-c, apo-III-b, and apo-III-a. It is also evident that the 17K protein is indistinguishable from apo-III-a in this system (Fig. 8, F, G).

Given that there are three molecular species of apo-III, it is important to know which apo-III becomes associated with lipophorin in response to AKH injection and, indeed, if all the apo-IIIs can associate. The lipophorin-free subnatant fraction obtained after density gradient centrifugation of hemolymph from resting and AKH-injected male locusts was subjected to native-PAGE. The results demonstrate that both apo-III-b and apo-III-c disappear in the subnatant obtained from AKH-injected hemolymph (Fig. 8, A, B), thereby indicating the association of these two

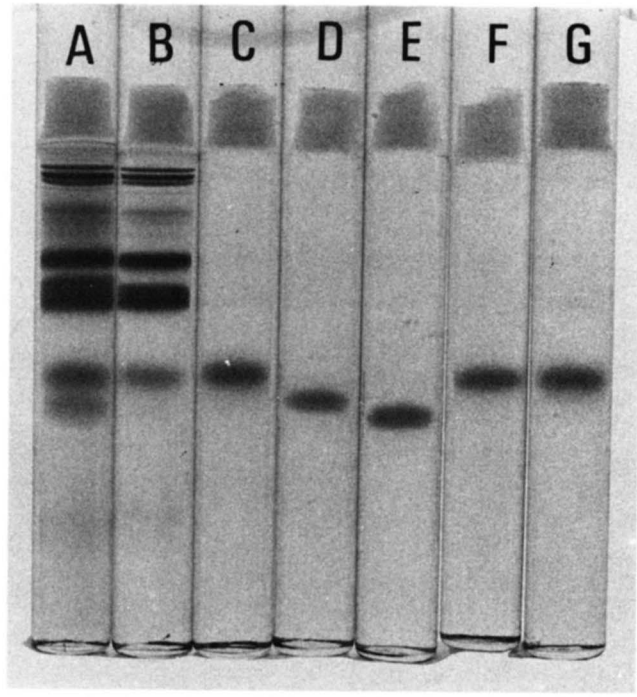

Fig. 8. Native-PAGE (10% gel) of apo-IIIs, 17K protein, and the lipophorin-free subnatant fraction from resting and AKH-treated hemolymph. A, Lipophorin-free subnatant (30 μg) from resting hemolymph; B, lipophorin-free subnatant from AKH-treated hemolymph (25 μg); C, apo-III-a (5 μg); D, apo-III-b (5 μg); E, apo-III-c (5 μg); F, 17K protein (5 μg); G, mixture of apo-III-a and 17K protein (2.5 μg of each).

apo-IIIs with lipophorin. However, the association of apo-III-a with lipophorin is not clear, because the 17K protein is not distinguished from apo-III-a on native gel and is always present in the subnatant fractions irrespective of the injection of AKH. In order to resolve this problem, activated lipophorin was run on urea-PAGE together with the three apo-IIIs for reference. The results are illustrated in **Fig. 9** and clearly demonstrate the presence of the three apo-IIIs in activated lipophorin, thereby indicating that all three apo-IIIs can associate with lipophorin in response to AKH injection.

The above observations, together with the elution profile of apo-IIIs on ion-exchange chromatography (Fig. 2), suggest that the three apo-IIIs have different electric charges. This possibility was tested by the use of isoelectric focusing to determine the isoelectric points of the three apo-IIIs. On the basis of data from three determinations, the isoelectric points were estimated to be pH 5.43 ± 0.04, 5.11 ± 0.03, and 4.98 ± 0.03 for apo-III-a, apo-III-b, and apo-III-c, respectively. These values are consistent with the elution profile of the three apo-IIIs on ion-exchange chromatography (Fig. 2) and the mobilities on native- and urea-PAGE.

How many molecules of apo-III associate with lipophorin?

Various amounts (weighed on a microbalance) of lyophilized apo-III-b were applied to SDS-PAGE and, after staining, the gels were scanned in a chromatographic scanner (Shimadzu, model CS-900) to provide a standard curve. A known amount (determined by protein analysis) of activated lipophorin was also run on SDS-PAGE, stained, and scanned. The amount of apo-III associated with lipophorin was then determined by reference to the standard curve. Since the molecular weights of heavy chain, light chain, and apo-III are 250K, 85K, and 20K, respectively, the number (x) of apo-III molecules associated with lipophorin can be calculated as

$$\frac{B}{A-B} = \frac{20x}{250 + 85}$$

where A represents the amount of activated lipophorin originally applied to the gel, and B represents the amount of apo-III associated with lipophorin as determined by the above procedure. Thus, the number of apo-III molecules associated with lipophorin was calculated to be 8 or 10/each lipophorin molecule; the value varied in accordance with the method by which the amount of activated lipophorin applied to gel was determined (8 by the Biuret method and 10 by the method of Lowry et al.).

The urea-polyacrylamide gel of activated lipophorin (Fig. 9) was further scanned to determine the molecular ratio of the respective apo-IIIs associated with lipophorin. The ratio was estimated to be 2:6:1 for apo-III-a:apo-III-b:apo-III-c. This ratio is consistent with the above values and, therefore, indicates that 2 moles of apo-III-a, 6 moles of apo-III-b, and 1 mole of apo-III-c associate with each mole of lipophorin in response to AKH injection.

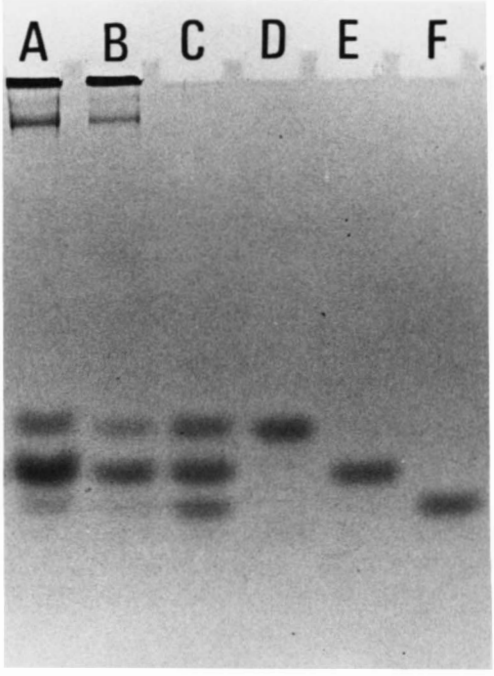

Fig. 9. Urea-PAGE (5% gel) of activated lipophorin and three apo-IIIs. A, Activated lipophorin (10 μg); B, activated lipophorin (5 μg); C, mixture of three apo-IIIs (2 μg of each); D, apo-III-a (2 μg); E, apo-III-b (2 μg); F, apo-III-c (2 μg). The heavy and light chains of activated lipophorin hardly migrate in this system.

Compositions of amino acid, amino sugar, and neutral sugar of three apo-IIIs

The amino acid compositions of three apo-IIIs are presented in **Table 1** and demonstrate that the three apo-IIIs are not distinguishable in terms of amino acid composition. The amino acid compositions of apo-III from *M. sexta* and 17K protein are also given in this table for comparison. It is evident that the amino acid composition of locust apo-III differs greatly from that of *M. sexta* apo-III (17). The most pronounced differences occur with methionine, tyrosine, and tryptophan; the former two are absent from locust apo-III while tryptophan is not present in the apo-III of *M. sexta*. Another striking difference is found in lysine; the content of this amino acid is very high in *M. sexta* apo-III but relatively low in locust apo-III.

A preliminary test for the presence of sugars in the three apo-IIIs using the anthrone method was positive and, therefore, the locust apo-III is a glycoprotein. Further analyses of sugar and amino sugar were carried out by gas–liquid chromatography and a colorimetric method. A typical chromatogram of neutral sugar from apo-III-b

TABLE 1. Amino acid compositions of locust apo-IIIs and 17K protein

Amino Acids	Apo-III-a	Apo-III-b	Apo-III-c	17K Protein	Apo-III of *M. sexta*[a]
			mol/1000 mol		
Asp/Asn	123	125	126	151	120
Thr	70	71	72	88	48
Ser	53	53	54	90	78
Glu/Glm	200	201	200	66	184
Pro	28	30	27	86	18
Gly	28	28	27	94	30
Ala	179	181	179	103	138
Val	49	49	50	74	60
Cys/2	1	1	1	11	0
Met	0	0	0	5	12
Ileu	36	37	38	40	12
Leu	106	108	110	53	72
Tyr	0	0	0	13	6
Phe	15	15	14	28	48
Lys	49	50	51	31	138
His	37	37	37	31	24
Arg	9	8	8	36	12
Trp	7	6	6	0	0

Data derived from analyses of duplicate samples.
[a] Modified from the data of Kawooya et al. (17).

is illustrated in **Fig. 10** and demonstrates the presence of fucose and mannose, but no other neutral sugars in appreciable quantity. **Table 2** presents quantitative data of the two sugars and indicates that the ratio of fucose to mannose amounts to about 1:3.5 for each of the three apo-IIIs.

Preliminary analysis of amino sugar composition by an amino acid analyzer revealed that glucosamine is the sole amino sugar found in the three apo-IIIs (data not shown). Therefore, the content of glucosamine was determined colorimetrically by the method of Smith and Gilkerson (29). The results are given in Table 2 and reveal that practically no difference exists among the three apo-IIIs, and that the glucosamine content is very high and exceeds the total amount of neutral sugars. Table 2 also indicates

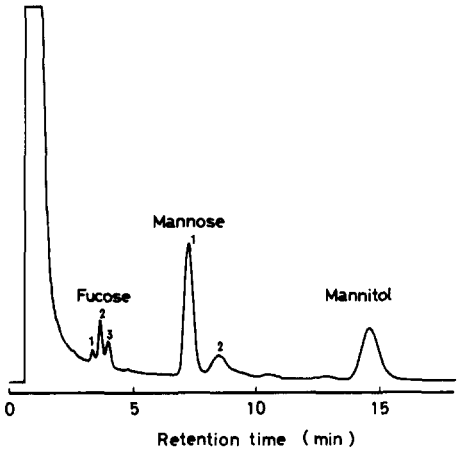

Fig. 10. Gas-liquid chromatogram of sugars associated with apo-III-b. The first peaks (1, 2, 3) and the second peaks (1, 2) were identified by mass spectrometry as fucose and mannose, respectively.

TABLE 2. The content of sugar and amino sugar in locust apo-IIIs

Apo-IIIs	Fucose	Mannose	Glucosamine
		%	
Apo-III-a	0.78 ± 0.06	2.73 ± 0.23	7.9 ± 0.2
Apo-III-b	0.77 ± 0.02	2.61 ± 0.22	8.2 ± 0.1
Apo-III-c	0.69 ± 0.07	2.64 ± 0.16	8.0 ± 0.1

The lyophilized (ammonium bicarbonate-free) apo-IIIs were weighed in a microbalance and analyzed by gas-liquid chromatography or by the colorimetric method of Smith and Gilkerson (29). The values represent the mean ± SD from three determinations and are expressed as the percentages of the weight of apo-III.

that the sum of neutral sugar and glucosamine amounts to approximately 11% of the total weight of apo-III, and that there is little difference among the three apo-IIIs.

Native molecular weights of three apo-IIIs

The lyophilized apo-IIIs were dissolved in 0.1 M NaCl and the molecular weights were determined by a rapid sedimentation-equilibrium method (26). The specific partial volume was theoretically calculated (32) from the amino acid composition (Table 1) and estimated as 0.73. The molecular weights of the three apo-IIIs were similar and estimated to be 19,100 ± 400, which is almost equivalent to the molecular weight (20,000) determined by SDS-PAGE, thereby indicating that locust apo-IIIs exist in hemolymph as monomers.

DISCUSSION

Van Der Horst et al. (13) have partially purified and characterized C_2-protein, which is equivalent to apo-III in this paper, from locust hemolymph. However, they used only gel filtration on Ultrogel AcA 22 or Sephacryl S-200 to isolate this protein and, therefore, it is difficult to compare the present data on apo-III with those reported for C_2-protein; however, the amino acid composition of C_2-protein is similar to that of apo-III as presented in Table 1.

The present study provides several lines of evidence for the existence of three molecular species of apo-III in locust hemolymph. The three apo-IIIs are indistinguishable by SDS-PAGE, immunodiffusion, and in amino acid composition, but their isoelectric points differ and, therefore, they are separable by native- or urea-PAGE. What causes such a difference in isoelectric points? One possibility is that the sugar residues of the respective apo-IIIs are phosphorylated to different extents, resulting in the different isoelectric points. At present, however, this problem remains unresolved, and it is necessary to elucidate the structure of the sugar chains present in the apo-IIIs to answer these questions.

The current study also reveals that locust apo-III and *M. sexta* apo-III differ markedly in physico-chemical properties. The locust apo-III is evidently a glycoprotein and exists as a monomeric form, whereas the *M. sexta* apo-III is reported to be a non-glycosylated protein which exists as a dimeric form (17). The apo-IIIs from the two insect species also exhibit considerable differences in amino acid composition (Table 1) and in molecular weights as determined by SDS-PAGE; 20,000 for locust apo-III and 17,000 for *M. sexta* (17). Apo-III isolated recently from hemolymph of the mesquite bug, *Thasus acutangulus*, (33) resembles the *M. sexta* apo-III in physico-chemical nature, thus, interspecific differences may exist in the nature of apo-III.

In a previous study (18), we scanned SDS-gels of activated lipophorin by a chromatographic scanner to determine the amount of apo-III associated with lipophorin and, based on the assumption that the three apoproteins (heavy chain, light chain, apo-III) stain equally on the gel, calculated the number of apo-III molecules associated with lipophorin. We proposed that 8 moles of apo-IIIs associate with each mole of lipophorin. This value is nearly equivalent to that (9 moles) determined in the present study, thus suggesting that the calculation method based on the above assumption is useful for determination of the approximate values.

Locust apo-III contains fucose in addition to mannose and glucosamine. This apo-III appears to be the first fucose-containing glycoprotein isolated from insect tissues, although such glycoproteins are common in vertebrate systems (34). Of particular interest is the very high content of glucosamine in locust apo-III which exceeds that of neutral sugar (Table 2), suggesting a complicated structure of the sugar chain. In most animal glycoproteins, the content of amino sugars is lower than, or at most equal, to the content of neutral sugars (34). Thus, the locust apo-III seems to be a unique glycoprotein. It is possible that the sugar chains play important roles as the recognition sites in the association of apo-III with lipophorin in response to the action of AKH. These possibilities await further investigation.

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