Further characterization of locust low density lipophorin induced by adipokinetic hormone

Eriko Nagao and Haruo Chino[1]

Biochemical Laboratory, Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan

Abstract This study was designed to resolve basic questions concerning the nature of low density lipophorin (LDLp) which is induced by adipokinetic hormone (AKH). For this purpose, lipophorin was fractionated by density gradient ultracentrifugation and each fraction containing lipophorin was analyzed for diacylglycerol and associated apolipophorin-III (apoLp-III). The diacylglycerol content of LDLp fractions increased significantly as the density of the fraction decreased (116 μg/100 μg protein at a high density to 209 μg/100 μg protein at a lower density). On the other hand, the content of diacylglycerol in each fraction of HDLp remained almost constant (33 μg/100 μg protein). It was also found that the number of apoLp-III molecules associated with LDLp increased as the density decreased (from 6.9 mol/mol LDLp to 13.2 mol/mol LDLp). However, electron microscopic observation showed that LDLp particles in each of the fractions were extremely heterogeneous in size with diameters of 29.4 ± 6.8 nm, 27.1 ± 5.5 nm, and 26.3 ± 5.7 nm for low, medium, and high density fraction, respectively. HDLp particles were very homogeneous in size irrespective of the fraction (15.9 ± 1.5 nm, 15.6 ± 1.5 nm, and 15.6 ± 1.3 nm for the respective fractions). A theoretical analysis based on all the experimental data strongly supports the hypothesis that the heterogeneity in the size of LDLp particles does not reflect different densities, but rather, heterogeneity is the result of intermolecular fusion between LDLp particles of the same density.—**Nagao, E., and H. Chino.** Further characterization of locust low density lipophorin induced by adipokinetic hormone. *J. Lipid Res.* 1991. **32:** 417–422.

Supplementary key words apolipophorin-III • diacylglycerol • electron microscopy • molecular size

Lipophorin, the major lipoprotein in the hemolymph of insects, serves as a reusable shuttle to transport various lipids including hydrocarbon, diacylglycerol, and cholesterol between tissues (1). Locust lipophorin exists in the hemolymph as high density lipophorin (HDLp) which is composed of two apoproteins, apolipophorin-I (apoLp-I, molecular weight 250,000) and apolipophorin-II (apoLp-II, molecular weight 85,000). However, under the influence of injected adipokinetic hormone (AKH), which is also known to be released from the corpora cardiaca during long distance flight (2), HDLp (d 1.12 g/ml) is transformed to low density lipophorin (LDLp, d 1.065 g/ml) due to uptake of diacylglycerol by lipophorin from the fat body (3). The formation of locust LDLp is also accompanied by the association of a third and lower molecular weight apoprotein, apolipophorin-III (apoLp-III, molecular weight 19,500) with lipophorin. Furthermore, we have previously reported that LDLp particles (average diameter 21.7 ± 3.0 nm) are larger and highly heterogeneous in size compared to that of the HDLp particles (14.5 ± 1.6 nm) (3). These structural changes of lipophorin induced by the injection of AKH into adult locusts have also been demonstrated recently in vitro by incubating purified HDLp and apoLp-III with dissected fat body in the presence of AKH (4). However, some basic questions concerning the nature of LDLp have yet to be completely resolved. How many molecules of apoLp-III associate with lipophorin? Why are the LDLp particles heterogeneous in size? In fact, we have reported that locust LDLp induced by the injection of AKH contains 9 mol of apoLp-III per mol of LDLp (5), while another research group (6) has proposed that 14 mol of apoLp-III associate with each mol of LDLp. The present study was designed to resolve these questions concerning the properties of AKH-induced locust LDLp.

MATERIALS AND METHODS

Animals and collection of hemolymph

Adult locusts, *Locusta migratoria*, fed on orchard and pampas grass and bran, (3–5 weeks after final molt) were taken from colonies maintained in this laboratory. Hemolymph was collected from adult locusts using the flushing method by injecting EDTA-containing saline into the hemocoel (7).

Abbreviations: LDLp, low density lipophorin; HDLp, high density lipophorin; AKH, insect adipokinetic hormone; apoLp-III, apolipophorin-III; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

[1]To whom correspondence should be addressed.

Chemicals

Locust AKH, a blocked decapeptide, was purchased from Peninsula Laboratories (San Carlos, CA). Chromatographically pure monostearin was obtained from Sigma (St. Louis, MO). All other chemicals were of analytical grade. Double-glass-redistilled water was used throughout.

Isolation of lipophorin

The freshly collected hemolymph was centrifuged for 5 min at 8000 *g* (4°C) to remove hemocytes. High density lipophorin (HDLp) was isolated from resting adult males by subjecting the hemolymph to KBr density gradient ultracentrifugation essentially according to Shapiro, Keim, and Law (8) as described by Chino, Downer, and Takahashi (3). To avoid contamination with other proteins, especially free apoLp-III, the yellow HDLp fraction was ultracentrifuged a second time according to the same procedure. Using the same method, low density lipophorin (LDLp) was isolated from the hemolymph of adult males, 90 min after injection of locust AKH (20 pmol/animal). After ultracentrifugation, the contents of the tubes were collected in 0.3-ml fractions from the top, and each fraction was monitored at 455 nm. The fractions containing lipophorin were analyzed for protein, diacylglycerol content, and apoLp-III, and were also examined by electron microscopy.

Preparation of apoLp-III and delipidated lipophorin

ApoLp-III was prepared essentially according to the method developed in this laboratory (5). The apoLp-III fraction obtained by the first chromatography on an Ultragel ACA 44 column was applied again to the same column to remove contaminating protein such as the 17-K protein (5). The rechromatographed apoLp-III fraction was dialyzed several times against distilled water, and finally lyophilized.

The HDLp was purified by a specific precipitation method (9). The solution of HDLp was first treated with 14 vol of chloroform–methanol 2:1 (v/v). The precipitate was collected by centrifugation and washed twice with chloroform–methanol 1:1 (v/v). The precipitate was then washed three times with 10% trichloroacetic acid to remove contaminating inorganic salts, and then washed once with acetone, and subsequently three times with ethyl ether to remove trichloroacetic acid. The precipitate was dried completely with a gentle air stream. The final preparation (white powder) as well as the above lyophilized apoLp-III were used as standard proteins for protein determination (see below).

Protein determination

The amount of protein was determined by the method of Lowry et al. (10). It was necessary to use delipidated lipophorin and pure apoLp-III, instead of bovine serum albumin, as standard proteins in order to determine the absolute protein amount of LDLp (a mixture of apoLp-I, apoLp-II, and apoLp-III). Only then could the exact number of apoLp-III molecules that associate with LDLp be calculated. Furthermore, the standard curves drawn with delipidated HDLp and purified apoLp-III indicated that there is no significant difference between the two absorbance curves at 750 nm, particularly at low concentrations. This is a great advantage and it allows calculation of a more exact amount of apoLp-III that is associated with lipophorin.

Determination of diacylglycerol

A known amount of monostearin was first added to each lipophorin fraction as internal standard. The lipids were then extracted with isopropanol–heptane 4:1 (v/v) (11) and the diacylglycerol content was analyzed by Iatroscanner (model Th-10), essentially according to Ackman (12) as described by Chino, Kiyomoto, and Takahashi (4). The rods (Chromarod S-III), to which the samples had been applied, were developed with benzene–methanol 60:1.5 (v/v) for 30 min.

Gel electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) of lipophorin was performed according to the method of Laemmli (13) and the gels were stained with Coomassie blue. The stained gel was scanned using a chromatographic scanner (Shimadzu TLC scanner CS-900).

Electron microscopy

Lipophorin preparations isolated by density gradient ultracentrifugation were first dialyzed against insect Ringer's solution (120 mM NaCl, 15 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), pH 7.0), and the samples (50 μg protein/ml) were applied to supporting films stained with 1% uranyl acetate, and observed in a JEOL JEM-1200EX electron microscope. The supporting films coated with carbon were made hydrophilic by ion bombardment before use.

RESULTS AND DISCUSSION

In a previous study, LDLp was characterized using the whole LDLp fraction (darkest yellow band) collected directly by a Pasteur pipet from the tube after KBr density gradient ultracentrifugation (3). Therefore, there was a possibility that the fraction collected by this method was composed of lipophorin particles having different densities. Thus, in the present experiment, hemolymph collected from the AKH-injected locusts was subjected to

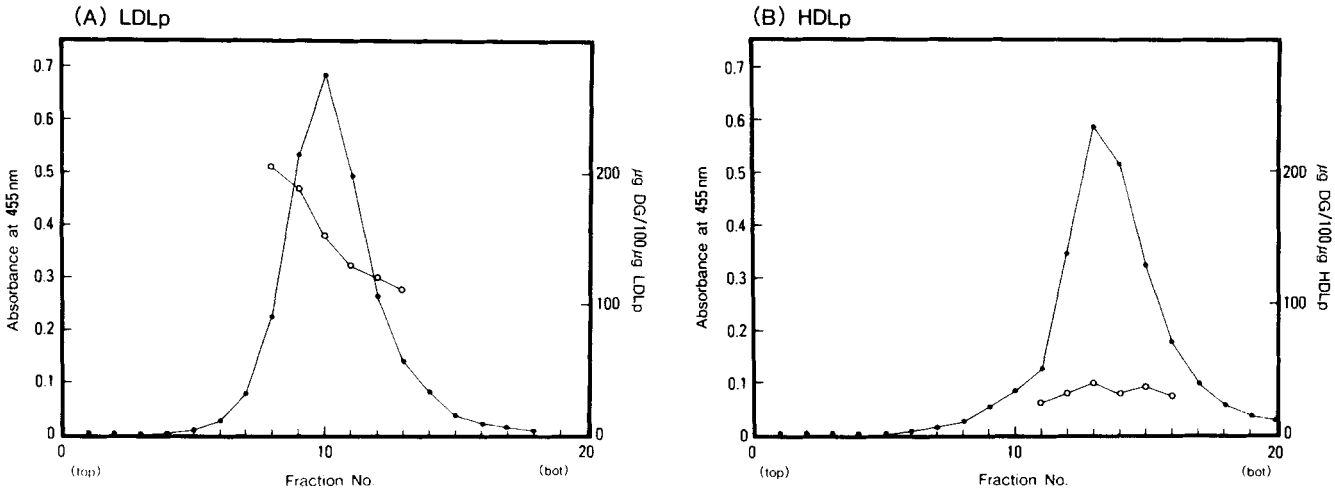

Fig. 1. Density gradient ultracentrifugation profile of low density lipophorin (LDLp) and high density lipophorin (HDLp), and the amount of diacylglycerol associated with each fraction of the two types of lipophorin. Hemolymph, collected from locusts without injection or 90 min after injection of AKH (20 pmol), was subjected to a second KBr density gradient ultracentrifugation. After centrifugation, the contents of tubes were fractionated in 0.3-ml fractions from the top. The yellow band (due to carotenoid pigments associated with lipophorin) was monitored at 455 nm. Diacylglycerol content and protein were determined in each fraction. Diacylglycerol values are expressed as mean of three experiments. A: LDLp; B: HDLp; solid circles, absorbance at 455 nm of a typical ultracentrifugation and fractionation run; open circles, amount of diacylglycerol.

KBr density gradient ultracentrifugation. After ultracentrifugation, the contents of the tubes were fractionated in 0.3-ml fractions from the top and each fraction containing lipophorin (monitored at 455 nm) was analyzed for diacylglycerol content and amount of apoLp-III associated.

Diacylglycerol content of LDLp and HDLp

Fig. 1 illustrates the density gradient profiles of LDLp (A) and HDLp (B), and also the content of diacylglycerol in each fraction of the two types of lipophorin. It was found that the diacylglycerol content of LDLp increased significantly as the density of the fraction decreased: from 116 μg/100 μg protein in the higher density fraction (no. 13) to 209 μg/100 μg protein in the lower density fraction (no. 8). By contrast, the content of diacylglycerol in each fraction of HDLp was almost constant (33 μg/100 μg protein), indicating that the density of each HDLp fraction is almost the same. Fig. 1 also demonstrates that the LDLp peak is almost as sharp as the peak of HDLp, indicating that the difference in the diacylglycerol content between lower and higher density fractions does not affect the sharpness of the peak. The prolonged ultracentrifugation (18 h) did not result in a significant change of the profile, although the LDLp peak became slightly broader and was slightly shifted to a lower density (data not shown).

Amount of apoLp-III associated with LDLp

In order to determine how many molecules of apoLp-III associated with lipophorin, a known amount of LDLp (determined by the method of Lowry et al. (10) using delipidated HDLp or apoLp-III as standard protein) was analyzed by SDS-PAGE. Various amounts of pure and lyophilized apoLp-III were also applied to the same gel to provide a standard curve. After staining, the gels were scanned. The amount of apoLp-III associated with LDLp was then determined by reference to the standard curve. Calculations were made according to the procedure described previously (5).

The results based on the above calculations are illustrated in **Fig. 2** and demonstrate that the number of

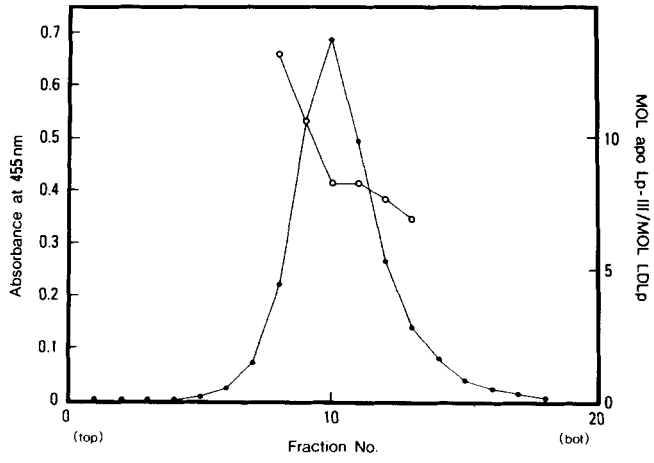

Fig. 2. Density gradient ultracentrifugation profile of low density lipophorin (LDL_p) and molar ratio of apoLp-III against LDL_p in each fraction. Hemolymph collected from locusts 90 min after injection of AKH was subjected to a second KBr density gradient centrifugation. After centrifugation, the contents of tubes were fractionated in 0.3-ml fractions from the top. (See also legend to Fig. 1.). The amount of protein in each fraction was determined, and the molar ratio of apoLp-III to LDLp was calculated as described by Chino and Yazawa (5). Solid circles, absorbance at 455 nm of a typical ultracentrifugation and fractionation run; open circles, molar ratio of apoLp-III to LDLp; each point represents the mean value from seven experiments (see text).

TABLE 1. Amounts of apoLp-III and diacylglycerol found in each fraction of LDLp

Fraction No.	ApoLp-III[a] (A)	Diacylglycerol[b] (B)	Ratio (B/A)
	mol	*mol*	
8	13.2	2014	153
9	10.7	1684	157
10	8.3	1254	151
11	8.3	1071	129
12	7.7	976	128
13	6.9	880	127

[a]Values are expressed as mol of apoLp-III/mol LDLp.
[b]Values are expressed as mol of diacylglycerol/mol LDLp (taking into consideration the amount of apoLp-III in the calculation of molecular weight of LDLp in each fraction).

apoLp-III molecules increased as the density of the fraction decreased: 13.2 ± 0.4 mol/mol LDLp in the lower density fraction (no. 8) and 6.9 ± 0.6 mol/mol LDLp in the higher density fraction (no. 13). If we were to combine the darkest yellow LDLp fractions, i.e., nos. 9–11, and calculate the amount of apoLp-III, the value would be 8.9 mol/mol LDLp. This value is consistent with the value proposed in the previous report, i.e., 9 mol/mol LDLp (5). The highest amount observed in fraction no. 8, however, is in accordance with the value (14 mol) proposed by Van der Horst et al. (6). We therefore conclude that, in addition to the existence of LDLp with the number of apoLp-III molecules as high as 14 mol/mol and as low as 7 mol/mol, the major population of LDLp particles induced in vivo by the injection of AKH contains about 9 mol apoLp-III/mol of LDLp.

A more detailed relationship between the contents of apoLp-III and diacylglycerol in LDLp fractions is shown in **Table 1**. The data demonstrate that the amount of apoLp-III associated with LDLp increases almost in parallel with the increase of diacyglycerol content in LDLp. Table 1 also shows that the molar ratio of diacylglycerol to apoLp-III is almost constant throughout the fractions and about 130–150 mol of diacylglycerol are added to lipophorin per mol of apoLp-III added. Wells et al. (14) reported that in the case of LDLp induced by AKH injection into the adult *Manduca sexta*, the molar ratio of diacylglycerol to apoLp-III was about 70 in lower density fraction (calculated from their data). This ratio is very low compared to the value shown in Table 1.

Although the physiological role of apoLp-III has not yet been completely elucidated, Kawooya et al. (15) have proposed that apoLp-III from *M. sexta* plays a role in increasing the capacity to accept more diacylglycerol by stabilizing the increased lipid–water interface resulting from diacylglycerol uptake. Furthermore, Wells et al. (14) have provided some data that suggest that apoLp-III is added to lipophorin particles more rapidly than diacylglycerol; the association of apoLp-III with lipophorin may function as a rate-limiting factor to regulate the rate of diacylglycerol uptake by lipophorin from the fat body. However, the problem of why apoLp-III, always present in hemolymph, does not bind spontaneously with lipophorin in the resting state remains an important question to be resolved. Concerning this problem, we have demonstrated that at least AKH has no direct effect on the association of apoLp-III with lipophorin (4, 16).

Electron microscopy of LDLp and HDLp

LDLp particles in the lower, medium, and higher density fractions, were observed by electron microscopy after negative staining with uranyl acetate. As a reference, HDLp particles were also observed. The results are illustrated in **Fig. 3**. The LDLp particles were heterogeneous in size in all the three fractions (see Fig. 3, A, B, C), whereas HDLp particles were highly homogeneous in size in all fractions (see Fig. 3, D, E, F). The histograms of distribution of the sizes of the LDLp particles are given in **Fig. 4**. The mean diameters of LDLp were calculated to be 29.4 ± 6.8 nm, 27.1 ± 5.5 nm, and 26.3 ± 5.7 nm for lower, medium, and higher density fractions, respectively, indicating that the population of larger size LDLp particles increases slightly as the density decreases. On the other hand, the mean diameters of HDLp were 15.9 ± 1.5 nm, 15.6 ± 1.5 nm, and 15.6 ± 1.3 nm for the corresponding fractions, respectively. This confirms the high degree of homogeneity in size of HDLp particles irrespective of the density of the fraction.

By using the following formula, we should be able to calculate the theoretical volume ratio of LDLp to HDLp.

$$\frac{V_{LDLp}}{V_{HDLp}} = \frac{(600{,}000 + x + y) / z}{600{,}000 / 1.12}$$

where V represents the volume of LDLp or HDLp, x represents the weight of added diacylglycerol per mol of LDLp, y represents the weight of added apoLp-III per mol of LDLp, z represents the density of LDLp, 600,000 is the molecular weight of HDLp (3, 9), and 1.12 is the density of HDLp (g/ml). If we apply the empirical values of x, y, and z obtained for each LDLp fraction in the present study to the above formula, the volume ratios of LDLp to HDLp are calculated to be 2.53, 1.90, and 1.61 for the low, medium, and high density fraction, respectively. Accordingly, the ratio of LDLp diameter to HDLp diameter (diameter is proportional to $V^{1/3}$) can be calculated to be 1.36, 1.23, and 1.17 for the respective density fractions. Thus, taking the average diameter of HDLp to be 15.7 nm, the diameters of LDLp in the three

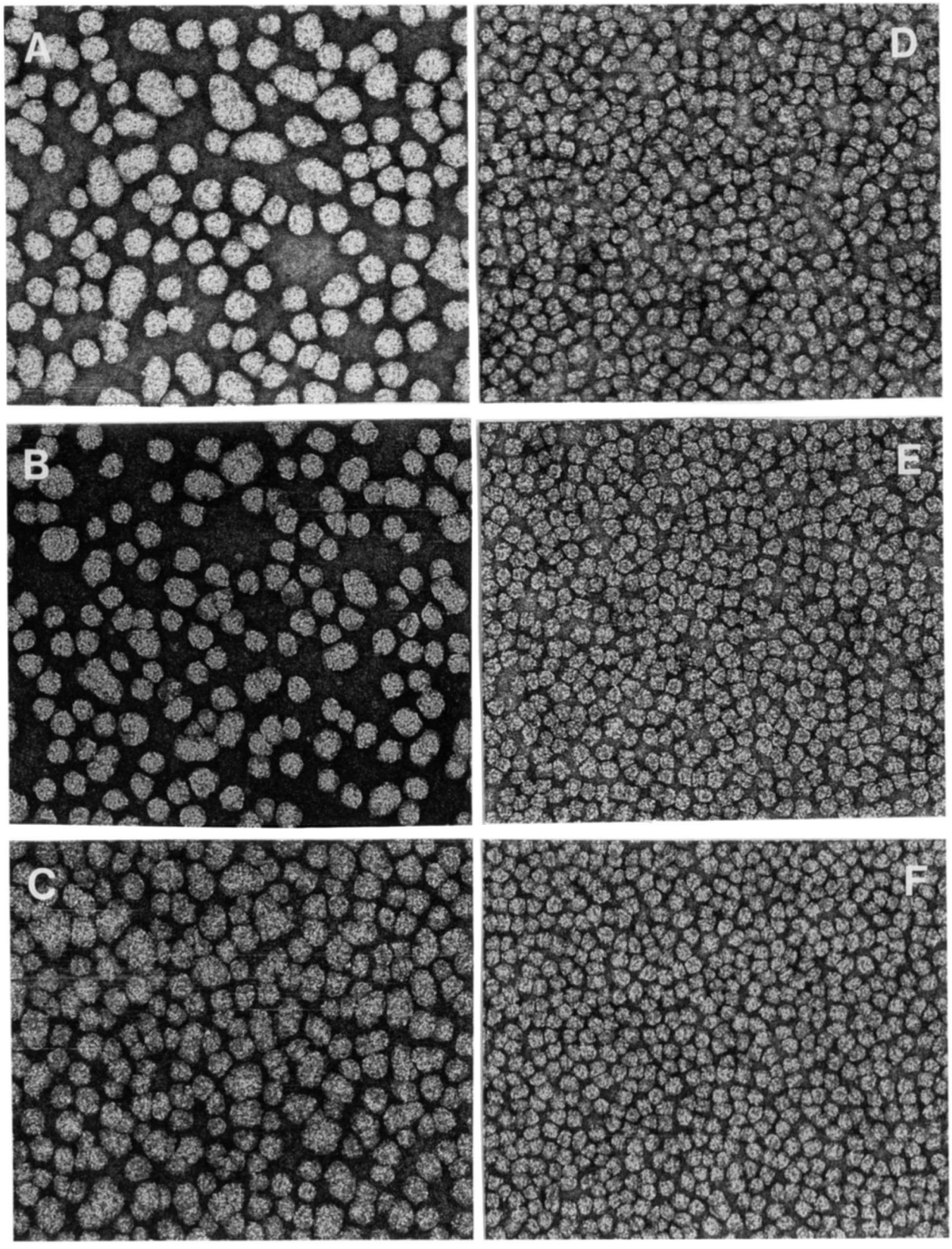

Fig. 3. Electron micrographs of lipophorin particles. LDLp and HDLp in the three different fractions were negatively stained with uranyl acetate (× 200,000). A: LDLp (fraction 8 as illustrated in Fig. 1A); B: LDLp (fraction 10); C: LDLp (fraction 13); D: HDLp (fraction 11 in Fig. 1B); E: HDLp (fraction 13); F: HDLp (fraction 15).

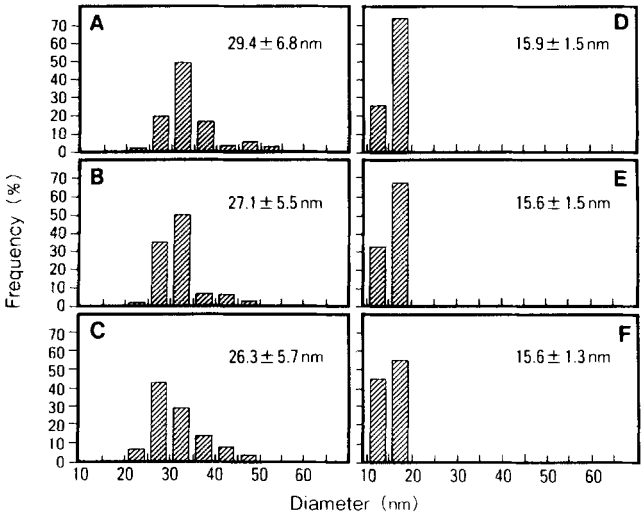

Fig. 4. Diameter of HDLp and LDLp particles. Values are mean ± SE of 240 particles within a specific region of the electron micrograph at a magnification of 200,000. A, B, C, D, E, and F represent A, B, C, D, E, and F in Fig. 3, respectively.

fractions should be 15.7 nm × 1.36 = 21.4 nm, 15.7 nm × 1.23 = 19.4 nm, and 15.7 nm × 1.17 = 18.4 nm, for low, medium, and high density fractions, respectively. Assuming that the size of LDLp particles reflects the amount of diacylglycerol loaded, then theoretical sizes of LDLp particles would be more or less the same as the sizes observed by electron microscopy. However, most of the LDLp particles observed in the three fractions far exceed the above calculated size of LDLp in the respective fraction. Some LDLp particles observed in all three fractions even had diameters larger than 45 nm (see Fig. 4). If we assume that such large heterogeneous LDLp particles with different size result from intermolecular fusion of different numbers of LDLp particles, we can resolve the apparent discrepancy between the heterogeneity in size and the homogeneity in density of LDLp; intermolecular fusion can then explain the fact that the ratio of protein to lipid is the same for each fused particle, irrespective of the size of the LDLp particles formed by fusion. Indeed, the electron micrograph (Fig. 3, A, B, C) shows some oval-shaped images that may represent the process of such fusion. Moreover, the increase in the mean diameter of LDLp particles as the amount of diacylglycerol and apoLp-III becomes higher (Fig. 4) implies that the LDLp with higher content of lipid and apoLp-III has a greater tendency to fuse intermolecularly. We believe that the discrepancy between the homogeneity in density and the heterogeneity in size observed for LDLp can reasonably be interpreted only by intermolecular fusion, and that such size heterogeneity is the most distinct characteristic of locust LDLp particles. However, the physiological significance and the mechanism of the intermolecular fusion of LDLp particles remain unknown.

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