A Strategy for Solubilizing Delipidated Apolipoprotein with Lysophosphatidylcholine and Reconstitution with Phosphatidylcholine†

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ABSTRACT: The apolipoproteins of insect lipophorin were dissociated in guanidinium chloride and isolated by gel permeation chromatography. Over 98% of the total lipid in lipophorin was associated with apolipophorin I (apoLp-I), thus suggesting this apolipoprotein to be the lipid binding component of the particle. ApoLp-I was delipidated with ethanol/ether and solubilized in buffer that contained radioactive lysophosphatidylcholine ([^{3}H]LPC) above the critical micellar concentration. Sonic irradiation of radioactive phosphatidylcholine ([^{14}C]PC) with [^{3}H]LPC-solubilized apoLp-I at a molar ratio of 318 resulted in reconstituted lipophorin I (RLp-I). [^{3}H]LPC was bound to fatty acid free bovine serum albumin and was separated from RLp-I by density gradient ultracentrifugation and gel permeation chromatography. Negatively stained RLp-I particles were quasispherical with an average radius of 55 Å, and their overall morphology and secondary structure were similar to those of native hemolymph lipophorin. The RLp-I particle had a ρ = 1.137 g/mL, a $M_r \approx 5.2 \times 10^5$, and a [^{14}C]PC:apoLp-I molar ratio of 308. From the compositional analysis, molecular size, trypsinization, and lipolysis with phospholipase A_2, we concluded that each RLp-I particle contained one molecule of apoLp-I and a monomolecular layer of [^{14}C]PC. When injected into the hemolymph of adult moths in vivo, RLp-I was loaded with lipid, as judged by a decrease in its density both in the presence and in the absence of adipokinetic hormone. The similarities in morphology and immunology of RLp-I and native lipophorin, together with the ability of RLp-I to load lipid, suggest that reconstituted lipophorins may serve as models to probe lipophorin structure and function.

Lipophorins are lipoproteins which transport lipids in insects (Shapiro et al., 1988). The molecular organization of the lipophorin components is not fully understood. Katagiri et al. (1987) have proposed a structural model for lipophorin that is reminiscent of the human lipoprotein model (Verdery & Nichols, 1975; Shen et al., 1977; Edelstein et al., 1979; Lund-Katz & Phillips, 1986). The human low-density lipoprotein (LDL)[1] model proposed by Lund-Katz and Phillips (1986) presents the particle as a spherical "oil-droplet" in which the bulk of the apolar lipid, mostly triacylglycerol and cholesterol esters, forms the core. The hydrophobic core is shielded from the surrounding aqueous medium by a monomolecular layer of phospholipids and some cholesterol as well as the apolipoprotein B-100. In contrast to mammalian lipoproteins, the hydrophobic core of insect lipophorin is said to consist of small amounts of cholesterol, triacylglycerol, and hydrocarbon. The surface of the particle is considered to be stabilized in the aqueous medium of the hemolymph by a monolayer of phospholipid, some diacylglycerol, and portions of the two apolipoproteins, apolipophorin I (apoLp-I, $M_r \approx 2.5 \times 10^5$) and apolipophorin II (apoLp-II, $M_r \approx 8.0 \times 10^4$). The bulk of the diacylglycerol is said to be situated between the hydrophobic core and the monomolecular layer of the phospholipid (Katagiri, 1985; Katagiri et al., 1987).

Unlike mammalian lipoproteins, insect lipophorin is postulated to function as a reusable lipid shuttle which transports lipid from the fat body (an organ with combined functions analogous to those of mammalian liver and adipose tissue) to sites of lipid utilization (Chino & Kitazawa, 1981). In the tobacco hornworm, *Manduca sexta*, apoLp-I and apoLp-II (which exist in a stoichiometric ratio of 1:1) and a small amount of phospholipid form the basic matrix of lipophorin (Ryan et al., 1986; Prasad et al., 1986; Tsuchida & Wells, 1988). Although lipid exchange occurs between lipophorin particles, the two apolipoproteins do not exchange (Ryan et al., 1986). Varying amounts of lipid may be mobilized in the fat body and added to, or removed from, lipophorin to form particles of different sizes and densities (Shapiro & Law, 1983; Prasad et al., 1986; Ryan et al., 1986; Kawooya et al., 1988). Lipid mobilization in *M. sexta* fat body is stimulated by a nonapeptide hormone, adipokinetic hormone (AKH) (Ziegler & Schulz, 1986). When moths are injected with AKH, large amounts of neutral lipid are released from the fat body and are associated with the high-density adult lipophorin (HDLp-A, $M_r \approx 7.63 \times 10^5$, ρ = 1.076 g/mL) to form the lipid-enriched low-density lipophorin particle (LDLp, $M_r \approx 1.56 \times 10^6$, ρ = 1.030 g/mL). The stability of LDLp in the hemolymph is reenforced by a third apolipoprotein, apolipophorin III (apoLp-III, $M_r \approx 1.8 \times 10^4$) (Kawooya et al., 1984, 1986a; Wells et al., 1987; Cole et al., 1987) which binds to the hydrophobic surface of the LDLp. ApoLp-III possesses unique properties which enable it to associate reversibly with LDLp (Kawooya et al., 1986a). The high solubility of apoLp-III in aqueous buffers has facilitated the analysis of its unique physical and surface properties (Kawooya et al.,

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[1] Abbreviations: LDL, low-density lipoprotein; apoB-100, apolipoprotein B-100; apoLp-I, apolipophorin I; apoLp-II, apolipophorin II; apoLp-III, apolipophorin III; HDLp-A, adult high-density lipophorin; LDLp, low-density lipophorin; GdmCl, guanidinium chloride; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; RLp-I, reconstituted lipophorin; [^{35}S]RLp-I, radiolabeled-reconstituted lipophorin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; CMC, critical micellar concentration.

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1984, 1986a). Unlike apoLp-III, insect apoLp-I and apoLp-II are insoluble in aqueous buffers but dissolve in buffers with high concentrations of chaotropes such as guanidinium chloride (GdmCl) (Kawooya et al., 1984; Shapiro et al., 1984). In this respect, these apolipoproteins are similar to mammalian apoB-100 (Walsh & Atkinson, 1983). Due to the insolubility of the insect apolipoproteins, very little is known about their properties and function (Pattnaik et al., 1979; Shapiro et al., 1984).

Protein–protein, protein–lipid, and lipid–lipid interactions are the major forces which stabilize mammalian lipoprotein structure (Verdery & Nichols, 1975; Edelstein et al., 1979; Atkinson & Small, 1986). The similarities in the molecular organization of human lipoproteins and insect lipophorin suggest such forces to operate in the molecular stability of lipophorin. Therefore, exploration of these forces may lead to an understanding of lipophorin structure and function. As an initial step toward addressing this problem, this paper presents a strategy for solubilizing delipidated apoLp-I in aqueous buffer that contains lysophosphatidylcholine (LPC). The apolipoprotein is then reconstituted into a lipophorin with phosphatidylcholine (PC) followed by removal of LPC with fatty acid free bovine serum albumin (FAF-BSA). The reconstituted particle is characterized and tested for its potential to load lipid in vivo.

Experimental Procedures

Materials. *M. sexta* were raised as described previously (Prasad et al., 1986). The reagents were obtained from the following sources: phosphatidylcholine (PC, $M_r \approx 786$), lysophosphatidylcholine (LPC, $M_r = 524$), and phosphatidylethanolamine ($M_r \approx 743$), Avanti Polar Lipids, Inc., Birmingham, AL; 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine, ^{35}S-labeling reagent, ^3H_2O, and [^{3}H]methyl iodide, Amersham Corp., Arlington Heights, IL; Omnifluor, New England Nuclear, Boston, MA; methyl iodide and 1,4,7,10,13,16-hexaoxacyclooctadecane (18-Crown-6), Aldrich, Milwaukee, WI; phospholipase A_2 (from *Crotalus durissus*) and trypsin (from bovine pancreas), Boehringer Mannheim Biochemicals, Indianapolis, IN; fatty acid free bovine serum albumin (FAF-BSA, $M_r \approx 6.6 \times 10^4$), Sigma Chemical Co., St. Louis, MO; SDS-PAGE protein standards, Bio-Rad Laboratories, Richmond, CA; Sepharose CL-6B and Sephacryl S-300, Pharmacia LKB Biotechnology, Inc., Pleasant Hill, CA; sequenal-grade 8 M GdmCl, BCA protein assay reagent, and Coomassie brilliant blue R-250, Pierce, Rockford, IL.

General Methods. Protein was assayed according to the method of Smith et al. (1985), and the radioactivity was measured by liquid scintillation spectrometry. Polyacrylamide gel electrophoresis (Laemmli, 1970) under denaturing conditions (SDS–PAGE) was performed on continuous (5–15%) gradient acrylamide slab gels which were then stained with Coomassie brilliant blue R-250. Lipophorin was isolated from the hemolymph of approximately 200 larvae of *M. sexta* in the wandering stage (Ryan et al., 1986) by two consecutive ultracentrifugation steps using a standard KBr discontinuous density gradient (Shapiro et al., 1984) at 50000 rpm, 5 °C for 16 h, using a VTi 50 rotor. Purity of lipophorin was verified by SDS-PAGE. Densities were measured by refractometry (Kawooya et al., 1988), and double radial immunodiffusion (Ouchterlony, 1968) was performed as described previously (Kawooya et al., 1986b).

Isolation of the Apolipoproteins of Lipophorin. The lipophorin solution from the density gradient purification step was dialyzed against 0.02 M Tris-HCl buffer, pH 8.4, containing 0.1 M NaCl and 1 mM EDTA. Solid GdmCl was added to the solution at a final concentration of 8 M, and dithiothreitol was added to the solution at a final concentration of 2 mM. The mixture was stirred (25 °C, 30 min) and applied to a Sepharose CL-6B column (5.0 × 150 cm) that was eluted with 0.02 M Tris-HCl buffer, pH 8.4, containing 6 M GdmCl. The purity of the eluted apoproteins was ascertained by SDS-PAGE.

Delipidation of the Apolipoproteins. All solutions were maintained at 4 °C. Each apoprotein fraction [apoLp-I (the yellow fraction) and apoLp-II (the colorless fraction)] was dialyzed against distilled H_2O in order to concentrate the apoproteins by precipitation. The precipitated apoproteins were pelleted by centrifugation (1000 rpm, 15 min, 5 °C) and then resuspended in 2 mL of H_2O. Two milliliters of ethanol/ether (1:1 v/v) was added to the apolipoprotein suspension, and the mixture was vortexed (15 s). Three milliliters of ethanol/ether (1:3) was added to the mixture. The suspension was vortexed (4×) at 10-min intervals and centrifuged (1000 rpm). The organic phase was transferred to a vial, and the aqueous phase was discarded. The protein pellet was resuspended in 2 mL of H_2O and was extracted 3 times as described above. The final protein pellet was rinsed with H_2O (5×) and was dissolved in 5 mL of 0.02 M Tris-HCl buffer, pH 8.4, containing 8 M GdmCl. A sample (1 mg) of the delipidated apoLp-I was treated with chloroform/methanol according to the procedure of Bligh and Dyer (1956) in order to ascertain that total delipidation had been achieved by the previous multiple extraction with ethanol/ether. The contents in the organic phases from the two different procedures of lipid extraction were analyzed by thin-layer chromatography using hexane/ether/acetic acid (60:40:1). The lipid spots on the TLC plate were visualized with iodine vapor. One milligram of apoLp-I and 1 mg of larval lipophorin were delipidated (separately), and the lipid content of each sample was determined as a percentage of the initial weight of the sample.

Preparation of [^{3}H]PC. PC was radiolabeled in its choline moiety by reacting 5 mCi of C^3H_3I (specific activity = 85 Ci/mmol) and 5.15 μmol of CH_3I with 1.28 μmol of phosphatidylethanolamine, using 5.12 μmol of 18-Crown-6 as a catalyst. The reaction was performed in 2 mL of anhydrous benzene containing 2.56 μmol of K_2CO_3. The experimental conditions for the reaction were as described by Patel et al. (1979). The purity of [^{3}H]PC was analyzed by two-dimensional TLC, using chloroform/methanol/28% NH_4OH (65:25:5) in the first dimension and chloroform/acetone/methanol/acetic acid/H_2O (12:16:4:4:2) in the second dimension (Rouser et al., 1970). Tritium in PC was measured, and the specific activity was determined.

Preparation of [^{3}H]LPC. [^{3}H]LPC was obtained by hydrolyzing 5 μCi of [^{3}H]PC (specific activity = 105 mCi/mmol) and 60 μmol of PC with 5 mg of phospholipase A_2 (specific activity ≈ 200 units/mg). The reaction was performed in 0.01 M Tris-HCl, pH 7.2, containing 7 mM $CaCl_2$ and 0.16 M KCl according to the method of Saito and Hanahan (1962). The products of lipolysis were extracted (3×) with chloroform/methanol (2:1), and the purity of [^{3}H]LPC was analyzed by thin-layer chromatography as described above for [^{3}H]PC.

Minimum Amount of [^{3}H]LPC Required To Solubilize Delipidated ApoLp-I. Varied amounts (from 0 to 1.2×10^{-6} mol) of [^{3}H]LPC (specific activity = 0.13 Ci/mol) were added to a series of tubes each containing 6.9×10^{-10} mol of delipidated and GdmCl-solubilized apoLp-I, in a final volume of 1 mL. The mixture was dialyzed (6×, 3 h) against 0.01 M Tris-HCl buffer, pH 8.4, containing 0.1 M NaCl and 1.91×10^{-5} M [^{3}H]LPC. Light scattering of each sample was

measured at 400 nm and was used to determine the minimum amount of [^{3}H]LPC that was required to solubilize 1 mol of apoLp-I in GdmCl-free buffer.

Reconstitution of ApoLp-I with PC. Prior to reconstitution, 3.87×10^{-6} mol of [^{3}H]LPC was mixed with 1.6×10^{-8} mol of delipidated and GdmCl-solubilized apoLp-I. The solution was dialyzed against 0.01 M Tris-HCl buffer, pH 8.4, containing 0.1 M NaCl and 1.91×10^{-5} M [^{3}H]LPC. The mixture (~4 mL) was transferred to a 15-mL-capacity corex tube which had been coated with a thin film of 5.09×10^{-6} mol of [^{14}C]PC (specific activity = 0.22 Ci/mol). The sample was purged with N_2 gas (in order to minimize autoxidation of the lipid) and was sonicated in a Branson water bath sonicator (Branson Sonic Power Co., Denbury, CT) for 60 s at 25 °C, to disperse the [^{14}C]PC. The mixture was incubated in a water bath [40 °C, 30 min (Ritter & Scanu, 1977)] followed by sonic irradiation (16 min, 40 °C, under N_2 gas, power setting at a continuous mode of 30 W) using a Branson ultrasonifier that was fitted with a standard microtip titanium probe. During ultrasonication, the temperature of the reaction was maintained around 40 °C by immersing the Corex tube in ice/water for 30 s, at 5-min intervals. Light scattering of the sample before and after sonic irradiation was measured at 400 nm.

Removal of [^{3}H]LPC from Reconstituted Lipophorin (RLp-I). The sonic irradiated sample was centrifuged (15 000 rpm, 5 °C, 15 min), and the supernatant solution was incubated with 1.21×10^{-6} mol of FAF-BSA (at 25 °C for 30 min), followed by ultracentrifugation (50 000 rpm, 5 °C, 4 h, VTi 50 rotor) in a modified KBr density gradient. The gradient was prepared as described previously for the isolation of egg lipophorin (Kawooya et al., 1988). After centrifugation, 1-mL fractions were drawn from the tube (Kawooya et al., 1988). Samples from selected fractions were analyzed by SDS-PAGE and were also measured for density, total protein, [^{14}C]PC, and [^{3}H]LPC. Fractions which contained apoLp-I were pooled, incubated with 1.21×10^{-6} mol of FAF-BSA as described above, and subjected to gel filtration using a Sepharose CL-6B gel column (1 × 120 cm). The column was eluted with 0.01 M Tes buffer, pH 7.2, containing 0.1 M NaCl. Samples from selected fractions were analyzed as described above for the analysis of the fractions from the ultracentrifugation step.

Removal of Nonbound [^{14}C]PC from RLp-I. ApoLp-I fractions from the gel filtration column were pooled and subjected to density gradient ultracentrifugation in a standard KBr gradient (Shapiro et al., 1984) (50 000 rpm, 5 °C, 16 h, VTi 50 rotor). Samples in fractions drawn from the tube were analyzed for total protein, [^{14}C]PC, [^{3}H]LPC, and density. In addition, the molar ratios of the various components in the samples were determined.

Preparation of RLp-I and [^{14}C]PC Bilayer Vesicles in the Presence of ^3H_2O. RLp-I was prepared by sonic irradiation of LPC-solubilized apoLp-I with [^{14}C]PC in the presence of ^3H_2O. The ^3H_2O:[^{14}C]PC ratio was measured immediately after sonic irradiation. [^{14}C]PC single-bilayer vesicles were prepared by sonic irradiation of [^{14}C]PC in buffer containing ^3H_2O. The RLp-I particle and the single-bilayer vesicles were dialyzed (3×) against Tris-HCl buffer after which the ^3H_2O:[^{14}C]PC ratio in the particles was measured.

Molecular Size. The molecular size of RLp-I was measured by gel filtration on a calibrated Sephacryl S-300 column (0.9 × 270 cm) that was eluted with Tes buffer. The column was calibrated with the following native lipophorin standards: LDLp, $M_r \approx 1.56 \times 10^6$, HDLp-A, $M_r \approx 7.68 \times 10^5$ (Ryan et al., 1986); high-density larval lipophorin, $M_r \approx 6.4 \times 10^5$ (Pattnaik et al., 1979); very high density egg lipophorin, $M_r \approx 4.14 \times 10^5$ (Kawooya et al., 1988).

Lipolysis of RLp-I with Phospholipase A_2. A solution of RLp-I was dialyzed against 0.01 M Tris-HCl buffer, pH 7.2, that contained 0.16 M KCl and 7 mM $CaCl_2$. Lipolysis was initiated by preincubating a solution that contained 3.07×10^{-4} M RLp-I and 2.4×10^{-4} M FAF-BSA at 37 °C for 10 min under N_2 gas. Phospholipase A_2 was added to the solution at a concentration of 2.38×10^{-8} M (total volume = 3 mL). The enzyme was omitted in the control experiment. The solution was incubated for 1 h at 37 °C, followed by extraction (2×) with chloroform/methanol (2:1). Lipid components were analyzed by TLC, using a dichloromethane/methanol/H_2O (65:25:4) solvent system, and were visualized as described above. The lipid spots were scraped from the TLC plate, transferred to a vial containing 10 mL of 0.04% Omnifluor in toluene, and measured for radioactivity.

Treatment of RLp-I with Trypsin. Approximately 1.62×10^{-4} M RLp-I was incubated with 1.34×10^{-8} M trypsin in 0.05 M sodium phosphate buffer, pH 7.0, that contained 0.1 M NaCl (total volume = 3 mL), for 1 h at 37 °C. Trypsin was omitted from the control experiment. After incubation, samples were drawn from the incubation mixtures and were analyzed by SDS-PAGE.

Preparation of [^{14}C]PC Single-Bilayer Vesicles. Single-bilayer vesicles of [^{14}C]PC were prepared by sonic irradiation (1 h, 41 °C under N_2 gas) of 1.73×10^{-6} mol of [^{14}C]PC (specific activity = 0.22 Ci/mol) in 4 mL of 0.01 M buffer, pH 7.0, that contained 0.1 M NaCl. The sample was centrifuged (18 000 rpm, 5 °C for 30 min), and the supernatant solution was applied to a Sepharose 4B column (1 × 120 cm). The column was eluted with 0.01 M sodium phosphate buffer, pH 7.0, that contained 0.1 M KCl. The lipid in each fraction was measured by scintillation spectrometry. Fractions at the center of the peak of the eluted vesicles were pooled and were used for circular dichroic (CD) measurements.

Circular Dichroism. The CD spectra of RLp-I, native lipophorin, and [^{14}C]PC single-bilayer vesicles were measured on a Cary 60 spectropolarimeter that was equipped with electronics from Aviv Associates Inc. (Lakewood, NJ). The measurements were performed under a steady stream of N_2 gas, in 0.01 M sodium phosphate buffer, pH 7.0, that contained 0.1 M KCl. During the course of the experiment, the temperature was maintained at 25 °C. The potential contribution of [^{14}C]PC of RLp-I to the overall CD spectrum of RLp-I was deduced from measurements of the spectrum of the [^{14}C]PC single-bilayer vesicles. Prior to measurements, the single-bilayer vesicles were diluted with buffer to the same concentration as the [^{14}C]PC in the RLp-I. The CD spectrum of delipidated apoLp-I was measured in Sequenal-grade 8 M GdmCl at pH 7.0. All spectra were measured between 190 and 250 nm and were corrected for base-line contributions from the buffer solutions. The data were analyzed according to the method of Chang et al. (1978).

Preparation of [^{35}S]RLp-I. A solution containing 2.0×10^{-8} mol of delipidated apoLp-I in GdmCl was added to 3.78×10^{-6} mol of [^{3}H]LPC. The solution was dialyzed (3×, 2 h) against 0.1 M sodium borate buffer, pH 8.5, that contained 1.9×10^{-6} M [^{3}H]LPC. A sample (1.0×10^{-8} mol of apoLp-I) from the solution was incubated with 0.15 mCi of ^{35}S-labeling reagent (specific activity = 1.282×10^3 Ci/mol) for 40 min at 0 °C. The reaction was stopped by adding 0.5 mL of 0.5 M glycine, and the sample was dialyzed (6×, 16 h at 4 °C) against sodium borate buffer. After measurement of total

protein and radioactivity, the specific activity of the radiolabeled apoLp-I ([^{35}S]apoLp-I) was determined. This sample was used to prepare radioactive reconstituted particle ([^{35}S]RLp-I) exactly as described for the preparation of RLp-I.

Fate of Reconstituted Particles Injected into M. sexta. Eight male moths (12 h after emerging from pupae) were each injected with 0.45 μCi of [^{35}S]RLp-I (specific activity = 180 Ci/mol) in 20 μL of phosphate-buffered saline (0.1 M sodium phosphate, pH 7.0, that contained 0.15 M NaCl/1 mM EDTA, pH 7.0). Immediately after injection of the [^{35}S]-RLp-I solution, four of the moths were each injected with 10 pmol of *M. sexta* AKH (courtesy of Dr. Fernando-Warnakulasuriya, Department of Biochemistry, University of Arizona, Tucson, AZ), and the remaining four moths were each injected with 20 μL of phosphate-buffered saline. After 1 h, hemolymph was collected from each moth and was subjected to density gradient ultracentrifugation under the standard conditions (Shapiro et al., 1984). Forty fractions (1 mL/fraction) were drawn from each ultracentrifugation tube and were analyzed for radioactivity and density as described above for the analysis of these components in RLp-I.

Results and Discussion

Isolation of the Apoproteins of Lipophorin. M. sexta larval high-density lipophorin contains 63% protein and 37% lipid of which phospholipid and diacylglycerol predominate (Pattnaik et al., 1979; Shapiro et al., 1984; Prasad et al., 1986). The hydrophobic components of insect lipophorins (Katagiri et al., 1987), like those of mammalian lipoproteins (Morrisett et al., 1977; Shen et al., 1977), are assembled in the particle in such a way that they are excluded from the surrounding aqueous medium of the circulatory system. The molecular exclusion of these components from water epitomizes the hydrophobic effect which may have a great impact on the stability of lipophorin structure. The hydrophobic effect is readily disrupted by organic solvents (Scanu, 1966) and detergents (Helenius & Simons, 1971; Walsh & Atkinson, 1983) as well as chaotropes such as GdmCl (Kawooya et al., 1984; Shapiro et al., 1984). In the present study, we dissociated the apolipoproteins of lipophorin by a variation of the method of Shapiro et al. (1984). We used 8 M GdmCl at pH 8.4, 25 °C, in the presence of dithiothreitol, instead of 6 M GdmCl, pH 7.0, at high temperature. The apolipoproteins were isolated by gel permeation chromatography, during which apoLp-I was eluted as a yellow fraction and apoLp-II was a colorless solution. Both apolipoproteins were insoluble in aqueous GdmCl-free buffers. Figure 1 shows the apolipoproteins of lipophorin before and after isolation by column chromatography.

Distribution of Lipid in the Apoproteins. Until now, the distribution of lipid among the apolipoproteins of lipophorin was unknown. In the present study, we found the lipid to account for 38% by weight of larval lipophorin and for 49% by weight of isolated apoLp-I. No lipid was detectable in isolated apoLp-II. Calculations based on the above percentages, and involving corrections for the differences between the apolipoprotein composition of apoLp-I and that of larval lipophorin (larval lipophorin contains apoLp-I and apoLp-II), showed the total amount of lipid recovered from isolated apoLp-I to be similar to the total lipid that was extracted from the native larval lipophorin. Extensive washing of apoLp-I precipitate (after removal of GdmCl by dialysis) did not alter the lipid content of the apoLp-I fraction, thus indicating that the lipid was not free in the form of micelles, but was associated with the apolipoprotein. We conclude from these results that apoLp-I is the lipid-binding apolipoprotein of lipophorin,

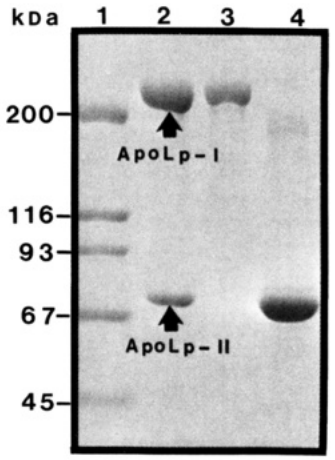

FIGURE 1: SDS–PAGE (4–15%) slab gel of lipophorins and isolated apolipoproteins. (1) Molecular weight markers; (2) lipophorin isolated from the hemolymph of fifth-instar larva by KBr density gradient ultracentrifugation; (3) isolated apoLp-I; (4) isolated apoLp-II. The apolipoproteins were dissociated from the native lipophorin particle and were isolated by gel permeation chromatography as described under Experimental Procedures.

and for this reason, it was the focus of the reconstitution studies. Whether apoLp-II serves some other function besides a potential structural role remains to be explored.

Delipidation of ApoLp-I Prior to Reconstitution. Although the apolipoproteins of lipophorin were dissociated from the particle by GdmCl, the evidence presented above shows that this chaotrope did not remove the lipid from apoLp-I. Therefore, prior to reconstitution, apoLp-I was delipidated by a variation of the method of Scanu (1966) which minimizes potential irreversible protein denaturation. Following delipidation, extraction of the sample by the more drastic method of Bligh and Dyer (1956) showed no lipid in the protein fraction, thus confirming total lipid removal from apoLp-I by repetitive extraction with ethanol/ether.

Solubilization of ApoLp-I in Buffer Containing GdmCl. When dissolved in buffer that contained 8 M GdmCl, the apolipoprotein formed a viscous solution at concentrations of >2 mg/mL. Addition of dithiothreitol prevented the formation of this viscous solution, thus suggesting the disruption of intermolecular disulfide bridges. Similar disulfide bridges have been reported in delipidated mammalian apoB-100 and are thought to induce the formation of high molecular weight aggregates of the apolipoprotein (Cardin et al., 1982).

Choice of Detergent. In the present study, we observed that unlike mammalian apoB-100 (Helenius & Simons, 1971; Walsh & Atkinson, 1983), apoLp-I could not be delipidated and solubilized by sodium deoxycholate or by other detergents which are suitable for reconstitution of membrane proteins (Hjemeland & Chrambach, 1984). From the general survey of the solubility of apoLp-I in buffers that contained various detergents, we observed that detergents which form large micelles (Triton X-100, $M_r \approx 9.0 \times 10^4$; LPC, $M_r \approx 9.5 \times 10^4$) (Helenius et al., 1979) were effective in solubilizing apoLp-I whereas those with smaller micelles (sodium deoxycholate, $M_r \approx 9.1 \times 10^3$; sodium cholate, $M_r \approx 2.1 \times 10^3$) were not effective in maintaining apoLp-I in a soluble form. Although LPC and Triton X-100 were effective in solubilizing apoLp-I, these detergents are generally not used for reconstitution because of their low CMC values (9.9×10^{-6} M for LPC and 3.0×10^{-4} M for Triton X-100), large micelle size, and the zwitterionic nature of LPC (Saunders, 1966; Helenius & Simons, 1975; Kupferberg et al., 1983). Due to the above properties, LPC and Triton X-100 cannot be separated from

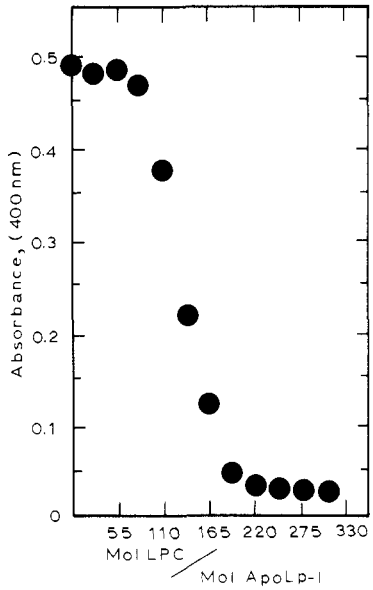

FIGURE 2: Solubility of delipidated apoLp-I in aqueous buffer that contained [^{3}H]LPC. A fixed amount (6.9×10^{-10} mol) of delipidated apoLp-I (solubilized in GdmCl) was mixed with varied amounts (0 to 1.2×10^{-6} mol) of [3H]LPC. The mixture was dialyzed against buffer that contained [^{3}H]LPC just above the CMC. After dialysis, light scattering of the samples was measured at 400 nm.

the reconstituted particle by dialysis, or by gel filtration.

Despite these potentially undesirable LPC properties, we used LPC to solubilize apoLp-I in GdmCl-free buffer because unlike Triton X-100 the structure of LPC is similar to its precursor, PC (a molecular thought to stabilize lipophorin in the aqueous medium). Furthermore, LPC also occurs in small amounts in native lipophorin (Pattnaik et al., 1979) and has been used to study the interactions between lipid and mammalian apolipoproteins (Verdery & Nichols, 1974; Gwyne et al., 1975). Unlike Triton X-100, we observed LPC not to interfere with the colorimetric protein assay or with protein measurements at 280 nm. Of most significance to this study is the ability of LPC to bind to FAF-BSA (Nishida, 1968; Pattnaik et al., 1976; Kupferberg et al., 1983), thus providing a means for its ultimate separation from RLp-I. This procedure could not be used to remove Triton X-100.

Solubility of ApoLp-I in GdmCl-Free Buffer That Contains [^{3}H]LPC. The solubility of apoLp-I in GdmCl-free buffer was dependent upon [^{3}H]LPC concentration in the buffer (Figure 2). In the absence of [^{3}H]LPC or the presence of low concentrations of the detergent, apoLp-I was precipitated when GdmCl was removed by dialysis, thus forming turbid solutions with high light-scattering values at 400 nm. However, at higher concentrations of [^{3}H]LPC, the resulting solutions clarified as indicated by the minimal light-scattering values (Figure 2). Once GdmCl was removed, the precipitated apoLp-I could not be resolubilized by the addition of [^{3}H]LPC. It was therefore necessary to maintain adequate amounts of [^{3}H]LPC in the solutions during dialysis. The amount of [^{3}H]LPC in the dialysis buffer was maintained at its CMC to ensure the presence of sufficient monomeric species of the detergent in equilibrium with the micelle species (Helenius & Simons, 1975). Such an equilibrium would prevent the loss of [^{3}H]LPC from the apoLp-I solution (in the dialysis bag) to the dialysis buffer. Figure 2 shows that in the presence of 220 mol of [^{3}H]LPC, apoLp-I solution remained clear when GdmCl was removed. This value was similar to the total number of LPC monomers (181) that occur in a single micelle of LPC (Saunders, 1966). From this observation, we conclude

Table I: Conditions for Reconstituting RLp-I[a]

component	RLp-I
[^{14}C]PC:apoLp-I[b]	318
[^{3}H]LPC:apoLp-I[b]	239
[^{3}H]LPC:[^{14}C]PC[b]	0.752
temp (°C)	40
pH	8.4
duration of sonic irradiation (min)[c]	16

[a] Reconstitution was performed as described under Experimental Procedures. [b] Expressed as molar ratio. [c] Reduces A_{400nm} from an initial value 1.132 to 0.038.

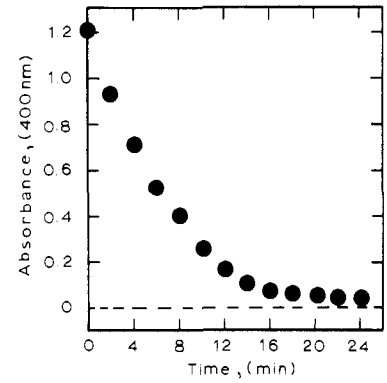

FIGURE 3: Solution clarification as a function of the time of sonic irradiation. [^{14}C]PC was mixed with [^{3}H]LPC-solubilized apoLp-I at a molar ratio of 318 and was subjected to sonic irradiation as described under Experimental Procedures. At selected intervals, light scattering of the solution was measured at 400 nm. Sonic irradiation of the mixture was discontinued when the light-scattering value was about 0.045.

that an estimated minimum of one micelle of LPC was required to solubilize 1 mol of apoLp-I. Therefore, this amount of [^{3}H]LPC was the minimum that was used routinely to solubilize apoLp-I, as excess [^{3}H]LPC would involve extra steps for its ultimate removal from the reconstituted lipoprotein.

Conditions for Incorporation of [^{14}C]PC into ApoLp-I. During the early stages of developing a method for reconstitution of lipoprotein, we observed that sonic irradiation of a fixed amount of apoLp-I with varied amounts of [^{14}C]PC produced lipid–protein particles of different sizes and densities. From these observations, we decided to reconstitute lipophorin particles with size and density similar to those of larval lipophorin. Table I shows the optimal conditions for reconstituting apoLp-I with [^{14}C]PC. Suspension of [^{14}C]PC in [^{3}H]LPC-solubilized apoLp-I at a molar ratio of 318 resulted in a turbid solution with a light-scattering value of 1.132 at 400 nm (Figure 3). The solution clarified progressively upon sonic irradiation, with ultimate clarity ($A_{400nm} \approx 0.038$) occurring within 16 min (Figure 3). The observed change from a turbid to a clear solution was probably due to the formation of a [^{14}C]PC–[^{3}H]LPC–ApoLp-I complex. In addition to the duration of sonic irradiation and the initial [^{14}C]PC:apoLp-I molar ratio, the formation of this complex was dependent upon the temperature and pH of the buffer solution (data not shown). Thus, like mammalian apoB-100 (Ginsburg et al., 1984), apoLp-I bound lipid more readily in the alkaline pH range than at neutral or acidic pH (data not shown).

Separation of [^{3}H]LPC and Nonbound [^{14}C]PC from the RLp-I Particle. The [^{3}H]LPC present in the [^{14}C]-PC–[^{3}H]LPC–apoLp-I solution was initially bound to FAF-BSA and was then separated from RLp-I by a combination of density gradient ultracentrifugation and gel permeation chromatography. Figure 4A,B shows the distribution of

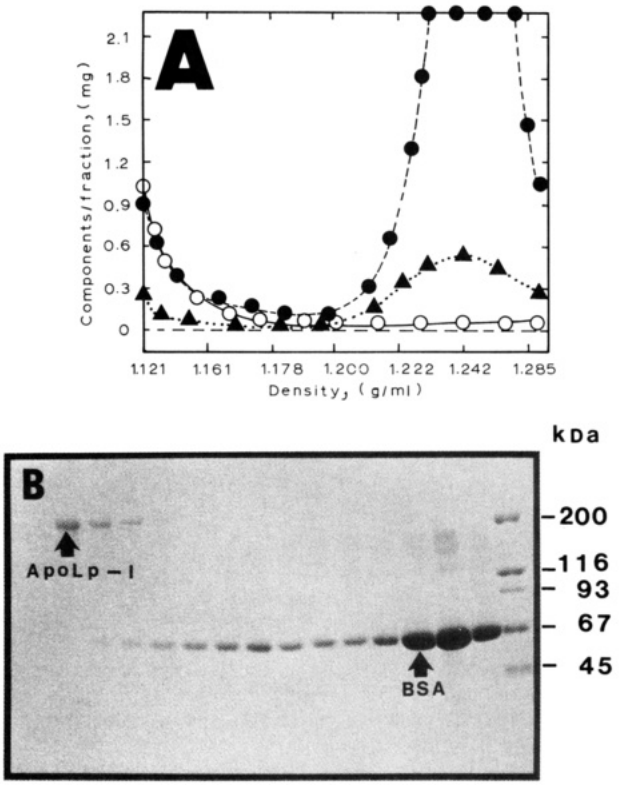

FIGURE 4: Removal of [^{3}H]LPC and separation of BSA from RLp-I by density gradient ultracentrifugation. After [^{3}H]LPC-solubilized apoLp-I was sonic irradiated with [^{14}C]PC, FAF-BSA was added to the solution, and the mixture was subjected to KBr density gradient ultracentrifugation as described under Experimental Procedures. (A) Samples drawn from individual fractions were analyzed for (●) total protein, (○) [^{14}C]PC, and (▲) [^{3}H]LPC. (B) Samples of selected fractions from the density gradient step were subjected to SDS–PAGE on a 4–15% slab gel.

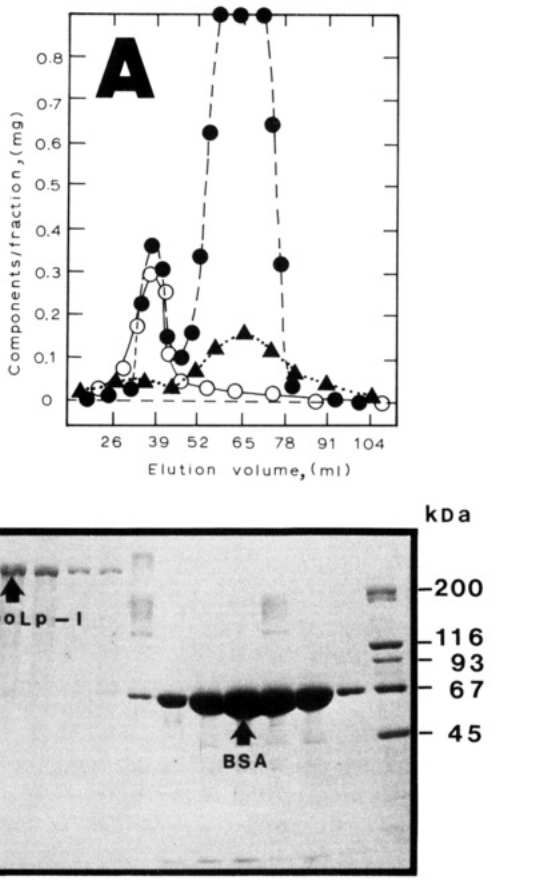

FIGURE 5: Removal of [^{3}H]LPC and separation of BSA from RLp-I by gel permeation chromatography. Fractions from the density gradient ultracentrifugation step (Figure 4) that contained apoLp-I were pooled and applied to a Sepharose CL-6B column. The column was eluted with 0.01 M Tes buffer, pH 7.2, that contained 0.1 M NaCl. Samples from selected fractions were analyzed for the distributions of lipid and protein. (A) Elution profile from gel filtration: (●) total protein; (○) [^{14}C]PC; (▲) [^{3}H]LPC. (B) SDS–PAGE (4–15%) of protein from selected fractions of the elution profile.

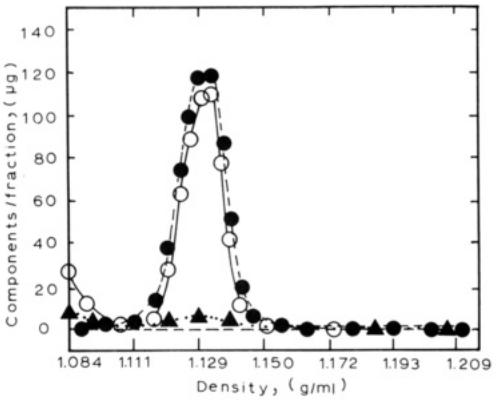

FIGURE 6: Equilibrium density gradient ultracentrifugation profile of RLp-I. Fractions from the gel permeation chromatography step were pooled and subjected to ultracentrifugation in a modified KBr density gradient as described under Experimental Procedures. The distributions of (●) protein, (○) [^{14}C]PC, and (▲) [^{3}H]LPC in the gradient were analyzed as described under Experimental Procedures.

[^{3}H]LPC, [^{14}C]PC, apoLp-I, and BSA in samples from selected fractions of the density gradient ultracentrifugation step. Both [^{14}C]PC and apoLp-I comigrated at the lower density end of the gradient, whereas BSA and most of the [^{3}H]LPC were in fractions at the high-density end. After ultracentrifugation, there was still a substantial amount of [^{3}H]LPC in the fractions that contained [^{14}C]PC–apoLp-I (Figure 4A). In order to reduce the remaining [^{3}H]LPC, the fractions containing [^{14}C]PC–apoLp-I were pooled, and the solution once again was incubated with FAF-BSA, followed by gel permeation chromatography. Figure 5A shows the elution profile of the components from the incubation mixture. The gel filtration method was effective in separating both FAF-BSA and [^{3}H]LPC from [^{14}C]PC–apoLp-I (Figure 5B). After the gel filtration step, nonbound [^{14}C]PC was separated from the [^{14}C]PC–apoLp-I complex (RLp-I) by a modified ultracentrifugation step. In this gradient, RLp-I migrated at a higher density than the nonbound [^{14}C]PC (Figure 6). The figure shows that most of the [^{14}C]PC was associated with apoLp-I. This ultracentrifugation step was also used to measure the equilibrium density of RLp-I.

Effectiveness of BSA and [^{3}H]LPC Removal. Calculations of the [^{3}H]LPC:[^{14}C]PC molar ratios at each step of the [^{3}H]LPC removal revealed the following results. Prior to the first ultracentrifugation step (Figure 4), the [^{3}H]LPC:[^{14}C]PC molar ratio in the [^{14}C]PC–[^{3}H]LPC–apoLp-I complex was 0.752. After this step, the [^{3}H]LPC:[^{14}C]PC molar ratio was reduced to 0.391. The second step, that involved gel filtration (Figure 5A), reduced the [^{3}H]LPC:[^{14}C]PC molar ratio further to 0.095. After the final step of the equilibrium density gradient ultracentrifugation (Figure 6), the [^{3}H]LPC:[^{14}C]PC molar ratio was 0.038. These values together with the SDS–PAGE analyses (Figures 4B and 5B) show that the method outlined above was effective in removing both [^{3}H]LPC and BSA from the [^{14}C]PC–apoLp-I complex.

Properties of Reconstituted Lipophorin. Table II shows the properties of RLp-I that include density, size, and chemical

Table II: Composition of Reconstituted Lipophorin I[a]

property	RLp-I
apoLp-I (mol)	1
[^{14}C]PC:apoLp-I[b]	308
[^{3}H]LPC:apoLp-I[b]	16
[^{3}H]LPC:[^{14}C]PC[b]	0.038
M_r (from gel filtration)	5.20×10^5
M_r (from chemical composition)	4.98×10^5
hydrated density (g/mL)	1.137

[a] The values were obtained from the experiments performed as described under Experimental Procedures. [b] Expressed as molar ratio.

Table III: Radius of RLp-I in Comparison with the Radii of Native Lipophorin[a]

lipophorin particle	radius (Å)
LDLp	84.4
HDLp-A	67.3
high-density larval lipophorin	56.6
RLp-I (from electron microscopy)	54.7
RLp-I (from gel filtration)	53.6
very high density egg lipophorin	51.0

[a] The radii of LDLp, HDLp-A, high-density lipophorin of the larva, and very high density egg lipophorin were calculated from the molecular weights of these particles using the equation presented under Discussion.

composition. RLp-I was eluted from the Sephacryl S-300 column as a narrow symmetrical peak (data not shown) and also formed a single symmetrical peak when subjected to density gradient ultracentrifugation (Figure 6), thus suggesting the particles to be relatively homogeneous. When RLp-I was subjected to gel filtration on the column that was calibrated with native lipophorin standards, the plot of $(V_e - V_0)/(V_t - V_0)$ vs log r [where V_e = elution volume, V_0 = void volume, V_t = total volume, and r = radius of the lipophorin standard (Table III)] was linear and was used to determine the radius of RLp-I, which we found to be 53.6 Å. We then used this radius in the equation $r = (3Mr/4\pi\rho N)^{1/3}$ (where r = radius of RLp-I, ρ = density of RLp-I, and N = Avogadro's number) to calculate the size of RLp-I, which we found to be $M_r \approx 5.20 \times 10^5$. This value was comparable to that of $M_r \approx 4.98 \times 10^5$ we calculated from the chemical composition of the particle (Table II).

The structure of RLp-I was examined by electron microscopy. Negatively stained RLp-I particles were quasispherical with an average diameter of 109 Å (Figure 7). This diameter was similar to the diameter (107 Å) of RLp-I particles that we determined from the linear plot of the radii of the native lipophorin standards. The electron micrograph (Figure 7) shows that RLp-I particles were homogeneous and similar in morphology to the native lipophorin particles that have been examined by a similar method (Pattnaik et al., 1979; Chino et al., 1986).

Organization of the Lipid in RLp-I. An RLp-I particle with a radius of 55 Å is expected to occupy a volume of 6.97×10^5 Å^3. This value is consistent with the volume of 7.2×10^5 Å^3 which a particle that contains 1 molecule of apoLp-I and 308 molecules of [^{14}C]PC would occupy. Calculations show that in such a particle, apoLp-I would occupy a volume of 3.07×10^5 Å^3, and [^{14}C]PC would occupy a volume of 4.07×10^5 Å^3. From these values, we deduced that a particle of this size and chemical composition was unlikely to be a protein–phospholipid bilayer vesicle. This was proven by the results from the experiments in which ^3H_2O was included in the buffers during the preparations of the RLp-I particle and [^{14}C]PC bilayer vesicles. In these experiments, the ^3H_2O:[^{14}C]PC ratio in the mixture prior to preparation of

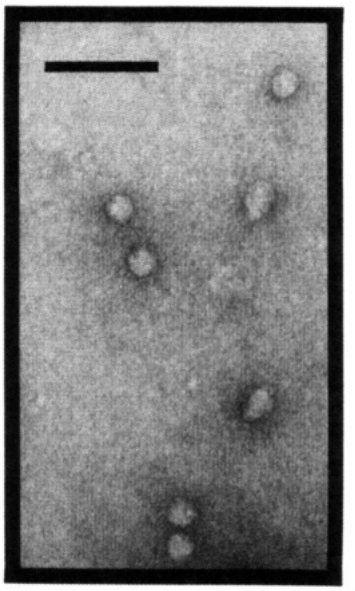

FIGURE 7: Electron micrograph of negatively stained RLp-I particles. RLp-I particles were applied in a droplet to a Formvar/carbon grid and were negatively stained with 2% uranyl acetate (pH 7.4). The particles were examined under a Siemens 101 electron microscope at an excitation energy of 80 kbar and 34000× magnification. Sizes of the particles were measured on enlarged photographs. Bar scale = 200 Å.

RLp-I was 0.567, but after dialysis of the isolated RLp-I, the ratio of the radioactive components was 0.000. In contrast, the ^3H_2O:[^{14}C]PC ratio prior to preparation of [^{14}C]PC-bilayered vesicles was 0.632. After extensive dialysis, the ratio was reduced to 0.259. Further dialysis of the vesicles did not alter this ratio. These experiments show that ^3H_2O was trapped in the [^{14}C]PC bilayer vesicles (as expected) but not in RLp-I particles, thus proving that RLp-I was not a vesicle. RLp-I was so susceptible to phospholipase A_2 that within 3 h of incubating the particle with the enzyme, more than 94% of the [^{14}C]PC was converted to [^{14}C]LPC. In addition the apolipoprotein of RLp-I was readily hydrolyzed by trypsin (data not shown). These results indicate that both apoLp-I and all of the [^{14}C]PC were directly in contact with the aqueous medium that surrounded the particle. Assuming that a single phospholipid molecule occupies an area of 68.5 Å^2 at the surface of the RLp-I particle and that all the [^{14}C]PC was located at the particle–water interface (since RLp-I is not a vesicle), then the particle may have consisted of an outer monomolecular shell of [^{14}C]PC that was permeated at various points by domains of hydrophilic amino acid side chains of apoLp-I. Assuming the shell of the particle to be 20 Å thick (which is the thickness of a PC monolayer), then the volume of this shell (assuming $\rho \approx 1.0$ g/mL for PC and $\rho \approx 1.35$ g/mL for protein) was 5.39×10^5 Å^3. One-fifth of the total volume of the shell was occupied by apoLp-I, and the remaining four-fifths of the volume was occupied by [^{14}C]PC. The volume of the core of the particle was 1.77×10^5 Å^3. One-third of the volume of apoLp-I was in the shell, and the remaining two-thirds of the volume was in the core. From the results presented above, we concluded that sonic irradiation of apoLp-I with [^{14}C]PC at a molar ratio of 318 led to the formation of a quasispherical protein–lipid complex with a core that probably consisted of domains of hydrophobic amino acid side chains of apoLp-I and an outer shell that consisted of a mixture of portions of apoLp-I and a [^{14}C]PC monomolecular layer.

Circular Dichroism. Treatment of proteins with chaotropes often leads to denaturation (Gordon & Jencks, 1963) that can

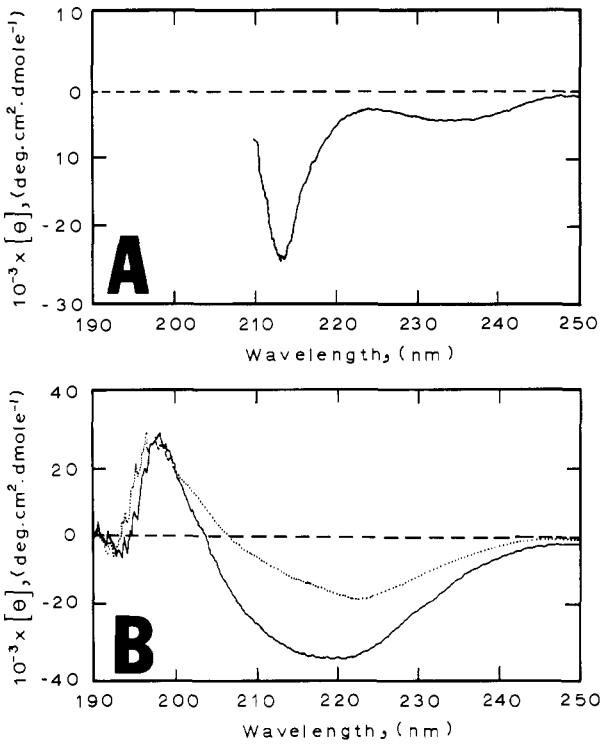

FIGURE 8: (A) Circular dichroic spectrum of delipidated apoLp-I. The CD spectrum of delipidated apoLp-I at a concentration of 0.085 mg/mL was measured in a 1-cm quartz cell as described under Experimental Procedures. (B) Circular dichroic spectra of reconstituted apoLp-I (dotted line) and native lipophorin (solid line). Circular dichroic spectra of RLp-I at a concentration of 0.064 mg/mL and native larval lipophorin at a concentration of 0.162 mg/mL were measured as described under Experimental Procedures.

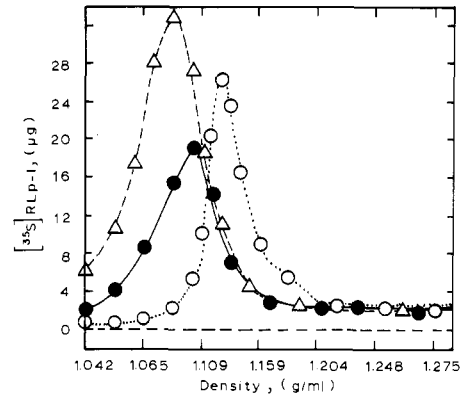

FIGURE 9: Changes in relative density of [^{35}S]RLp-I in vivo. Each adult moth was injected with 0.35–0.45 μCi of [^{35}S]RLp-I (specific activity = 180 Ci/mol). The moths were then divided into two groups of four. Five minutes later, each moth in one group was injected with 20 pmol of AKH in 20 mL of PBS, and in the other group, each moth was injected with 20 μL of PBS. After 1 h, the animals were bled, and the hemolymph from each animal (as well as the [^{35}S]RLp-I that was not injected in the animals) were subjected individually to KBr density gradient ultracentrifugation. The distribution of the radiolabel in the gradient was measured as described under Experimental Procedures. The density gradient profiles depict the following: (○) [^{35}S]RLp-I; (●) 1 h after injecting [^{35}S]RLp-I into control moths; (△) 1 h after injecting [^{35}S]RLp-I and AKH simultaneously into adult moths.

be examined by CD spectrometry (Gwyne et al., 1975) or by optical rotary dispersion spectrometry (Smith et al., 1972). In the present study, we examined the secondary structure of delipidated and GdmCl-solubilized apoLp-I as well as that of RLp-I. Figure 8A shows the CD spectrum of delipidated apoLp-I in 8 M GdmCl. The spectrum was characterized by an overall ellipticity with a minimum at 214 nm and a shoulder at 223 nm. Measurements of apoL-I ellipticity could not be made below 210 nm because of the high background readings that were attributable to GdmCl. The spectrum shown in Figure 8A represents a disordered structure similar to that of succinylated mammalian apoA-I (Gwyne et al., 1975) and to those of peptides (such as glucagon and calcitonin) which are known to possess minimal ordered structures (Edelhoch & Lippholdt, 1969; Brewer & Edelhoch, 1970).

The CD spectrum of RLp-I and larval lipophorin is shown in Figure 8B. The two spectra were similar in that each possessed an overall ellipticity with a maximum at 198 nm and minima between 217 and 222 nm. After five repetitive scans, the best estimate of the secondary structure of native lipophorin was 30% α-helix, 58% β-structure, and 12% random coil. The structure of RLp-I was 27% α-helix, 43% β-structure, and 30% random coil. Although the secondary structure of apoLp-I in native lipophorin is not known, the structure shown in Figure 8A suggests that the apoprotein was denatured by delipidation and treatment with GdmCl. However, on reassembly with [^{14}C]PC, apoLp-I acquired an ordered structure that was similar to but not identical with that of native lipophorin (Figure 8B). The [^{14}C]PC bilayer vesicles that were diluted to the same concentration as the [^{14}C]PC, that was associated with apoLp-I, did not show any contribution to the CD spectrum of RLp-I (data not shown). From these results, we conclude that denaturation of apoLp-I by delipidation and solubilization in GdmCl were reversible through reassembly of the apoprotein with [^{14}C]PC. A comparison of RLp-I and native lipophorin structures should be made with extreme caution, because unlike RLp-I, native lipophorin has apoLp-I and apoLp-II together with a variety of lipids of which diacylglycerol predominates.

Biological Activity of RLp-I. When tested with antibodies that were raised against native lipophorin, RLp-I formed a distinct immunoprecipitate band which fused with the immunoprecipitate band of native lipophorin (data not shown). Therefore, the various treatments which led to the reconstitution of the particle did not alter apoLp-I's antigenic properties. Attempts were made to determine if RLp-I had the potential to load lipid in vivo (as do native lipophorins) by measuring the density of [^{35}S]RLp-I before and after it was injected into the adult moths. The experiment was based on earlier studies which showed conclusively that hemolymph lipophorins undergo changes in densities that relate to known physiological events of lipid metabolism in vivo (Shapiro & Law, 1983; Wells et al., 1987). In the resting moths of *M. sexta*, 80% of the hemolymph lipophorin is HDLp-A, and the remaining 20% is LDLp (Wells et al., 1987; Kawooya & Law, 1988). During flight, however, most of the HDLp-A loads lipid from the fat body and is converted to LDLp. The transformation of HDLp-A to LDLp can be induced by injecting the moths with AKH (Shapiro & Law, 1983; Wells et al., 1987).

Figure 9 shows the apparent densities of [^{35}S]RLp-I before and after it was tested for lipid loading in vivo. [^{35}S]RLp-I had an apparent density of 1.123 g/mL. The density of the particle decreased to 1.102 g/mL 1 h after it was injected into the resting adult moths (Figure 9). When [^{35}S]RLp-I and AKH were injected simultaneously into the moths, the density of the particle decreased further from 1.123 to 1.088 g/mL (Figure 9).

In the resting animals, HDLp-A has an apparent density of 1.117 g/mL. When these animals are injected with AKH, most of the HDLp-A is converted to LDL-p with an apparent density of 1.052 g/mL (Kawooya et al., 1988). Ryan et al.

(1986) showed that insect hemolymph lipophorins do not exchange their apoLp-I or apoLp-II but are capable of exchanging their lipid components. The fact that the apparent density of [^{35}H]RLp-I in resting animals did not correspond to the density of either HDLp-A or LDLp shows the lack of exchange of [^{35}S]apoLp-I in RLp-I with the apoproteins of the hemolymph lipophorins. The data presented above show that although [^{35}S]RLp-I lacked apoLp-II, the particle did load lipid (in the animals that were not treated with AKH) as judged by a decrease in its density. In the presence of AKH, [^{35}S]RLp-I loaded lipid but less so than LDLp. It is probable that the absence of apoLp-II in [^{35}S]RLp-I and the presence of more [^{14}C]PC in the reconstituted lipophorin than is found in hemolymph lipophorins attenuated lipid loading by the particle. Although apoLp-II was soluble in buffer that contained [^{3}H]LPC, the apoprotein did not bind [^{14}C]PC. This observation is consistent with the lack of lipid in isolated apoLp-II.

The strategy presented here for solubilizing apoLp-I with [^{3}H]LPC and reconstitution with [^{14}C]PC adds a new tool for probing the structure of lipophorin. The observed similarities in the structures of RLp-I and native lipophorin as well as the ability of RLp-I to load lipid in vivo show that reconstituted lipophorins may serve as models for studies of lipid transport mechanisms in insects. Future studies will focus on the reassembly of apoLp-I with apoLp-II and various lipid components in order to determine how such reconstituted particles deliver lipids to cells. Comparison of reconstituted lipophorins with the native lipophorin particles could provide an insight on the intracellular assembly of the various lipophorin components. Since mammalian apoB, like insect lipophorin, is solubilized in blood by a monolayer of phospholipid, the reconstitution method presented here may be applicable to the reassembly of apoB with lipids.

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Hydrolysis of a Phospholipid in an Inert Lipid Matrix by Phospholipase A_2: A ^{13}C NMR Study†

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ABSTRACT: A new approach to study phospholipase A_2 mediated hydrolysis of phospholipid vesicles, using ^{13}C NMR spectroscopy, is described. [^{13}C]Carbonyl-enriched dipalmitoylphosphatidylcholine (DPPC) incorporated into nonhydrolyzable ether-linked phospholipid bilayers was hydrolyzed by phospholipase A_2 (*Crotalus adamanteus*). The ^{13}C-labeled carboxyl/carbonyl peaks from the products [lyso-1-palmitoylphosphatidylcholine (LPPC) and palmitic acid (PA)] were well separated from the substrate carbonyl peaks. The progress of the reaction was monitored from decreases in the DPPC carbonyl peak intensities and increases in the product peak intensities. DPPC peak intensity changes showed that only the *sn*-2 ester bond of DPPC on the outer monolayer of the vesicle was hydrolyzed. Most, but not all, of the DPPC in the outer monolayer was hydrolyzed after 18–24 h. There was no movement of phospholipid from the inner to the outer monolayer over the long time periods (18–24 h) examined. On the basis of chemical shift measurements of the product carbonyl peaks, it was determined that, at all times during the hydrolysis reaction, the LPPC was present only in the outer monolayer of the bilayer and the PA was bound to the bilayer and was ~50% ionized at pH ~7.2. Bovine serum albumin extracted most of the LPPC and PA from the product vesicles, as revealed by chemical shift changes after addition of the protein. The capability of ^{13}C NMR spectroscopy to elucidate key structural features without the use of either shift reagents or separation procedures which may alter the reaction equilibrium makes it an attractive method to study this enzymatic process.

Phospholipase A_2 (PLA_2)[1] catalyzes the hydrolysis of the ester bond at the *sn*-2 position of a diacylphospholipid, releasing fatty acid (FA) and lyso-1-acylphospholipid (De Haas et al., 1968). Its activity is found intracellularly as well as in cell secretions; in particular, extracellular forms of the enzyme are found abundantly in mammalian pancreas and in the venom of snakes and bees (Volwerk & De Haas, 1982). While its functional role in facilitating fatty acid turnover is well-known (Van Deenen, 1965), its regulatory roles in eicosanoid synthesis (McKean et al., 1981; Imai et al., 1982) and detoxification of phospholipid peroxides (Van Kuijk et al., 1987) have been discovered more recently. Intracellular PLA_2 activity in vivo is presumably normally well regulated to provide small amounts of substrates such as arachidonic acid while leaving the bilayer structure of the membrane intact.

It is clear from analyses of the kinetics of PLA_2 action that PLA_2 activity exhibits strong dependence on aggregation state, phase, and structure of zwitterionic phospholipids (e.g., diacylphosphatidylcholine; Dennis, 1983). However, as pointed out recently (Lister et al., 1988), a detailed kinetic analysis of PLA_2 data requires knowledge of structural features such as changes in the substrate structure during hydrolysis and the location, distribution, and partitioning of the hydrolyzed products in the presence and absence of albumin. Previous studies of the structural organization of phospholipid bilayers subjected to PLA_2 treatment have examined extensive hydrolysis. The results of Kupferberg et al. (1980) suggested that the entire outer monolayer of the vesicle could be hydrolyzed and that the inner monolayer was inaccessible to the

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[1] Abbreviations: BSA, bovine serum albumin; DHPC, dihexadecylphosphatidylcholine ether lipid; DPPC, dipalmitoylphosphatidylcholine; FA, fatty acid(s); LPPC, lyso-1-palmitoylphosphatidylcholine; PA, palmitic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA_2, phospholipase A_2.

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