Role of Diacylglycerol and Apolipophorin-III in Regulation of the Physicochemical Properties of the Lipophorin Surface: Metabolic Implications†

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ABSTRACT: *Manduca sexta* adult insects have two defined lipophorin species of densities 1.09 g/mL, [high-density lipophorin (HDLp)] and 1.02 g/mL [low-density lipophorin (LDLp)], respectively, and a continuous broad range of lipophorin particles of intermediate size and density, intermediate-density lipophorin (IDLp). The transformation of HDLp into IDLp and LDLp is the result of the progressive loading of HDLp with diacylglycerol (DG) and an exchangeable apolipoprotein, apolipophorin-III (apoLp-III). In this paper, we describe the physiochemical changes which occur in the lipophorin surface as a result of the transformation of HDLp into LDLp. (1) The increase in apoLp-III content, from 0 to 16 molecules per particle, is accompanied by a gradual increase in the ζ-potential which, at pH 8.6, ranges from +1.02 mV for lipophorins without apoLp-III to −7.76 mV for lipophorins containing 16 molecules of apoLp-III. (2) As judged by the changes in the partition constant for trimethylammonium diphenylhexatriene and oleic acid, an average 2-fold increase in the size of the lipophorin lipid surface takes place when HDLp is loaded with DG and transformed into LDLp. (3) These data, as well as the results obtained by end point lipolysis with a triacylglycerol (TG) lipase, indicated that the accessible DG content increases 4−7 times when HDLp is converted in LDLp. (4) Fluorescence polarization of the cationic and anionic lipid probes, trimethylammonium diphenylhexatriene and *cis*-parinaric acid, embedded in eight different subspecies of lipophorin, containing from 12 to 50% DG, showed a small decrease in the surface lipid order when going from HDLp (25% DG) to LDLp (50% DG). (5) Porcine pancreatic phospholipase A_2 was used as a probe of the lipoprotein surface. As the DG content of the lipoprotein increased, a higher enzyme activity against the lipoprotein−phospholipids was observed, with a maximum activity 5-fold higher against LDLp than against HDLp. Overall, the changes observed as the lipoprotein particles are loaded with DG and apoLp-III provide a link between the structure and properties of the lipophorin surface and the physiological roles of HDLp and LDLp particles.

Insects flying long distances use lipids to fuel flight (Beenakkers et al., 1984). Fat is stored in the fat body and mobilized mostly as diacylglycerol (DG),[1] which is carried by the hemolymph lipoproteins to the flight muscle. Lipophorin is the major lipoprotein found in the hemolymph of insects and transports phospholipid (PL), DG, hydrocarbons, sterol, and free fatty acids among the insect tissues. Lipophorins have been isolated from the hemolymph of many insect species, and these studies have been recently summarized (Shapiro et al., 1988; van der Horst, 1990; Blacklock & Ryan, 1994; Soulages & Wells, 1994). Lipophorin contains two molecules of apolipoprotein per particle: apoLp-I (250 kDa molecular mass), and apoLp-II (80 kDa molecular mass). *Manduca sexta* lipophorin is an excellent model system in which to study the physicochemical factors that govern the assembly and physiological functions of a lipoprotein because in the hemolymph of adult insects there are lipoprotein particles covering a broad density range, but the differences among the lipophorin subspecies are related to the variation of only two chemical components. A decrease in lipoprotein density is due to an increase in the DG -content, which, in turn, is accompanied by an increase in the content of a third exchangeable apolipoprotein, apoLp-III (18 kDa molecular mass). The simplicity of the system allows one to study the relationship between the composition and the physicochemical properties of the lipoproteins in a manner that is not possible with vertebrates' lipoproteins. In addition to the chemical simplicity, the physiological role of lipophorin subspecies from adult insects is quite well-defined, or predicted. For instance, under circumstances where the insect requires the mobilization of energy, as during flight, high-density lipophorin particles are loaded at the fat body with DG and apoLp-III (Wells et al., 1987; Nagao & Chino, 1991) and transformed into intermediate-density and low-density lipophorins. The role of IDLp and LDLp is to carry out a rapid transfer of fatty acid to other tissues.

The metabolism of a lipoprotein is primarily dependent on the properties of its surface. The surface domains of apolipoproteins control the interaction with receptors, which can mediate the internalization of the entire lipoprotein particle, as well as the exchange or hydrolysis of lipids by

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[1] Abbreviations: DG, diacylglycerol; PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; AKH, adipokinetic hormone; HDLp, high-density lipophorin; LDLp, low-density lipophorin; IDLp, intermediate-density lipophorin; apoLp-III, apoLp-II, and apoLp-I, apolipophorins-III, -II, and -I, respectively; TMA-DPH, trimethylammonium 1,3,6-diphenylhexatriene; cis-PnA, *cis*-parinaric acid; PLA_2, phospholipase A_2.

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the action of membrane-bound lipid carriers or lipases. On the other hand, the extent, chemical composition, hydration, charge, and the structural and dynamic properties of the lipid surface affect many of the processes involved in lipid transport and metabolism. The size of the surface lipid phase could affect the accessibility of certain lipolytic enzymes and lipid transfer proteins to their substrates. It could also modify the packing of the lipid phase by affecting lipid−protein interactions. The chemical composition of the surface lipid phase is clearly important, because the rate of desorption or hydrolysis of a component is proportional to its surface concentration.

In order to understand the biochemical basis of the physiological roles of different lipophorin subspecies, we studied some physicochemical properties of several lipophorin subspecies, and, in particular, the surface charge of different lipophorin subspecies and the size and lipid order of their surface lipid domains.

MATERIALS AND METHODS

Materials. The reagents were obtained from the following sources: *cis*-parinaric acid (cis-PnA) and trimethylammonium diphenylhexatriene (TMA-DPH), Molecular Probes, Eugene, OR; lipid standards, Avanti Polar Lipids, Inc., Alabaster, AL; Con A-sepharose and high-molecular mass markers, Pharmacia LKB, Inc., Piscataway, NJ; porcine pancreatic PLA_2, Sigma Chemical Co., St. Louis, MO; silica gel 60-precoated plates, J. T. Baker, Phillipsburg, NJ; BCA protein reagent, Pierce, Rockford, IL; and AKH, Peninsula Lab.

Insects and Hemolymph Collection. M. sexta were reared as previously described (Prasad et al., 1986). Larval hemolymph was collected through an incision in the second proleg directly into a bleeding solution [100 mM potassium phosphate buffer (pH 6.5) containing 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM diisopropyl phosphorofluoridate, and 10 mM glutathione]. The hemolymph from resting adult insects was obtained by decapitation of insects after the injection of 1 mL of the above bleeding solution. Hemolymph was also collected from adult insects 90 min after the injection of 100 pmol of AKH, which promotes lipid mobilization.

Purification of Lipophorin. Lipophorin was purified by ultracentrifugation in a KBr gradient, at 50 000 rpm, at 5 °C for 16 h using a VTi 50 rotor (Shapiro et al., 1984). Lipophorin of density 1.14 g/mL (HDLp-L) was obtained from the hemolymph of larvae that were in their feeding period. Lipophorins of densities 1.17 g/mL (HDLp-W1) and 1.11 g/mL (HDLp-W2) were obtained from insects that were in the first and second day of the prepupal stage, respectively. Lipophorin of density 1.17 g/mL was purified by two ultracentrifugation steps to eliminate non-lipoprotein contaminants. HDLp, IDLps, and LDLp were isolated from the hemolymph of adult insects. Lipoprotein densities were measured by refractometry.

Phospholipase Assay. ^{32}P-labeled lipophorin was obtained from the hemolymph of 2-day-old moths, 36 h after the injection of 100 μCi of carrier free $^{32}PO_4H_3$. The phospholipid-labeled lipophorin was kept in the KBr solution from the density gradient ultracentrifugation until the phospholipase assay was performed. The specific activity of the radiolabeled lipophorins was about 3.5×10^5 cpm/(μmol of PL). Lipophorin fractions were desalted twice by gel filtration on PD10 columns equilibrated with 0.1 M Tris-HCl buffer (pH 8.6) containing 2 mM EDTA and 0.01% NaN_3. To determine the initial rates of hydrolysis, the enzyme reactions were performed at 37 °C during 10 min and started by the addition of 0.15 unit (200 μL) of PLA_2 resuspended in 0.1 M Tris-HCl buffer (pH 8.6), containing 6 mM $CaCl_2$, to 200 μL of the lipophorin solution containing different amounts of lipoprotein. The reaction was stopped by addition of 50 μL of 1 M EDTA and 2.2 mL of chloroform−methanol (2/1 v/v). Then, 50 μL of 0.3 N HCl was added, and the phases were separated. The lower phase was taken to dryness, and after the addition of a carrier lipid mixture containing PC, PE, lysophosphatidylcholine (LPC), and lysophosphatidylethanolamine (LPE), the lipids were separated by thin layer chromatography (TLC) on silica gel G-precoated plates employing chloroform−methanol−28% ammonium hydroxide (65/25/5 v/v) as the developing solvent. Lipid spots were visualized by exposure of the plate to iodine vapor, and after the spots were marked, the iodine was allowed to evaporate. The spots were scraped from the plate and counted in a liquid scintillation counter. The spots corresponding to PC, PE, LPC, and LPE contained more than 90% of the radioactivity. The micromoles of phospholipid hydrolyzed was calculated from the lipophorin protein content and the number of phospholipid molecules per lipophorin particle. Because the molecular mass of the protein component of lipophorin changes with the content of apoLp-III, the apolipophorin composition was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and densitometry (see below).

Lipoprotein Lipase. The hydrolysis of the lipoprotein DG by the lipoprotein lipase from *Chromobacterium viscosus* (Wako Chemical) was determined colorimetrically employing the Triglyceride-G kit from Wako Chemical (Richmond, VA), which quantitates the production of glycerol that results from the enzymatic hydrolysis of the glyceride. Because the hemolymph of insects is known to contain free glycerol, the lipophorin samples were desalted twice against 50 mM Tris-HCl buffer (pH 7.5). The enzymatic reactions were carried out in duplicate at 37 °C for 20 min employing 120 units of enzyme and between 80 and 150 μg of lipoprotein in a reaction volume of 3.1 mL. Under these conditions, the reaction proceeded to completion and no further hydrolysis was observed after 10 min of reaction.

Electrophoresis. Samples were analyzed by SDS−PAGE in a 3.5−15% acrylamide gradient slab gel and stained with Commassie Blue R-250. The content of apoLp-III in lipophorin samples was determined by densitometric scanning of the wet gels employing a LKB Ultroscan XL densitometer.

Native gel electrophoresis of lipophorin samples was performed in 2.5−15% polyacrylamide gels (Nichols et al., 1986), at 4 °C, during 30 h at 130 V in Tris-borate buffer (pH 8.8). Size calibration was performed with Pharmacia high-molecular mass markers.

The electrophoretic mobility of lipophorin subspecies was determined by electrophoresis in 0.5% agarose gels (Beckman, Paragon Lipokit). Freshly prepared samples were desalted in PD10 columns equilibrated with 10 mM Tris-HCl buffer (pH 7.8) containing 0.15 M NaCl. Three to five microliters of sample, containing about 15 μg of protein, was loaded into the gels. Electrophoresis was performed for 30

min at 23 °C at 100 V. Gels were stained for lipids with Sudan Black and for proteins with Comassie Blue R250.

Fluorescence Measurements. Lipophorin samples were studied in 50 mM potassium phosphate buffer (pH 6.8) containing 0.1 M NaCl and labeled by addition of microliter aliquots of a solution of TMA-DPH in dimethyl sulfoxide (DMSO) or cis-PnA in ethanol. Fluorescence was determined in 1 cm path length cuvettes containing approximately 400 pmol of lipoprotein, determined by protein content with the BCA reagent employing BSA as standard, at a lipoprotein/probe ratio of 1/3. The suspension was incubated until fluorescence intensity reached a plateau. The concentrations of TMA-DPH and cis-PnA were determined spectrophotometrically using methanolic solutions of the probes and an extinction coefficient of 74×10^3 cm^{-1} M^{-1} at 365 and 320 nm for TMA-DPH and cis-PnA, respectively (R. P. Haugland, *Handbook of Fluorescent Probes and Research Chemicals*).

Anisotropy of fluorescence of TMA-DPH- or cis-PnA-labeled samples was measured in a Perkin-Elmer Model MPF 2A instrument. Excitation wavelengths were 365 and 320 nm (slit width = 8 nm) for TMA-DPH and cis-PnA, respectively. Emission was determined at 440 nm (slit width = 12 nm), for both probes. Incident scattered light was eliminated through a 390 nm cutoff filter. Unlabeled samples were used as reference blanks, which served to correct for light-scattering contributions to the fluorescence signal. The contribution of light scattering never represented more than 4% of the fluorescence signal. The polarization ratio is given by $P = GI_{\parallel}/I_{\perp}$, where $I_{\parallel}$ and $I_{\perp}$ refer to intensities emitted parallel and perpendicular, respectively, to the vertically polarized excitation beam and G is a factor for instrumental correction (Azumi & McGlyn, 1962). The steady-state fluorescence anisotropy (r_s) was calculated using the equation: $r_s = (P - 1)/(P + 2)$, where P is the polarization ratio previously defined.

Binding Studies. The binding of TMA-DPH to different lipophorin particles was studied at 25 °C, by measurement of the increase in fluorescence at 440 nm upon excitation at 365 nm. Stray light was reduced by a 390 nm cutoff filter. Microliter aliquots of a lipoprotein stock solution were added to a 0.7 μM solution of TMA-DPH in 50 mM potassium phosphate buffer (pH 6.8) containing 0.14 M NaCl. Blank corrections arising from the increase in the fluorescent signal due to light scattering and/or fluorescence of the lipoprotein never exceeded 0.5% of the fluorescence signal. Data were also corrected to account for the dilution produced after the addition of lipoprotein. Corrections due to the inner filter effect were not necessary. The binding equilibrium of TMA-DPH was described by the partition constant, K_p, defined as K_p = (moles of bound probe/moles of Lp)/(moles of free probe/moles of water]. In order to obtain the K_p values, the fluorescence enhancement data were fitted by nonlinear regression analysis to the equation $I = I_{max}\mathrm{Lp}/([\mathrm{Lp}] + 55.5K_p^{-1})$.

Electron Microscopy. Lipophorin samples were studied by transmission electron microscopy in a Phillips EM 420 microscope at an acceleration voltage of 80 kV. Lipoprotein was adsorbed onto carbon-coated grids by immersion of the grids into 50 μg/mL lipoprotein solutions. Samples were negatively stained by immersion of carbon-coated copper grids into a 1.5% solution of uranyl acetate in deionized water.

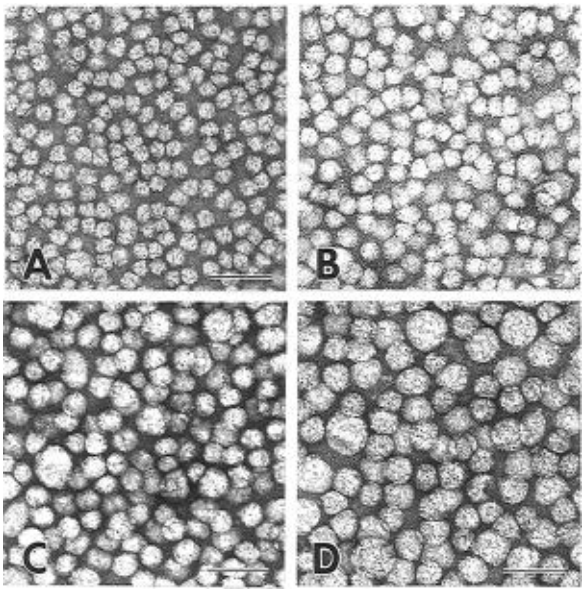

FIGURE 1: Electron Micrographs of negatively stained lipoprotein subspecies: (A) HDLp-A (d = 1.095 g/mL); (B) IDLp-1 (d = 1.07 g/mL), (C) IDLp-2 (d = 1.05 g/mL), and (D) LDLp (d < 1.04 g/mL). Bar scale = 50 nm.

RESULTS

Figure 1 shows the electromicrographs obtained with four adult lipoprotein fractions of different density. The increase in size that accompanies the decrease in density can be observed by a simple inspection of the particles. A considerable heterogeneity of the lipophorin particles is observed by electron microscopy, as well as by gradient gel electrophoresis (data not shown). The average radii, in angstroms, of the lipoproteins are as follows: HDLp, 70.5 ± 4.5; IDLp-1, 88.5 ± 17; IDLp-2, 99.5 ± 19; and LDLp, 124.5 ± 26. The sizes of the lipoprotein particles, as estimated by electron microscopy, are much larger than those obtained by analytical ultracentrifugation, where average diameters of 67.3 and 85.2 Å were obtained for HDLp and LDLp, respectively (7). The origin of these differences is not clear; however, the larger sizes estimated by electron microscopy could be due to flattening of the lipoprotein particles (6). The smallest particle fraction is HDLp, which contains about 200 molecules of DG and 2 molecules of apoLp-III. The largest particles are found in the LDLp fraction and contain about 1000 DG molecules and 16 molecules of apoLp-III (Wells et al., 1987).

Lipoprotein Surface Charge. The characterization of lipophorin subspecies by electrophoresis in agarose gels was performed following the method reported by Sparks and Phillips (1992). The variation of the mobilities with the density and apoLp-III content of the lipophorin particles is shown in Figure 2. Larval lipophorin subspecies that do not contain apoLp-III, with a density range of 1.17−1.11 g/mL, showed positive mobilities at pH 8.6. The positive charge of the lipophorin particles that lack apoLp-III is consistent with the absence of negatively charged lipid in lipophorin and the fact that lipophorin does not bind to anionic exchange resins.

On the other hand, lipoprotein particles containing apoLp-III, with a density range of 1.09−1.03 g/mL, show an increasing negative charge as the apoLp-III content of the lipophorin increased from 2 to 16 molecules per particle.

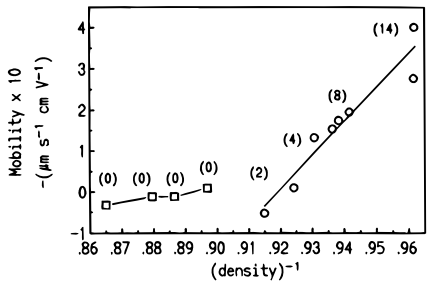

FIGURE 2: Electrophoretic behavior of different lipophorin subspecies. Electrophoretic mobility of larval (□) and adult (○) lipoproteins of different density. The apoLp-III content of the different lipoproteins is included in parentheses.

Following the method described by Sparks and Phillips, we estimated that in this density range the electrostatic potential and the valence of the lipophorins vary from +1.02 to −7.76 mV and from −1.7(−e) to 18.9(−e), respectively.

In adult lipophorins, the only compositional changes that accompany decreases in density are an increase in apoLp-III content and an increase in DG content. Therefore, the changes in mobility, or electrostatic potential, can be attributed to the increase in apoLp-III content, and the changes in the observed particle charge are consistent with the properties of apoLp-III, which has a p*I* of 5.7 and an estimated net charge of 4.7(−e) at pH 8.6.

Size of the Lipophorin Lipid Surface. Because TMA-DPH binds to the lipoprotein surface (Ben-Yashar & Barenholz, 1991) and assuming that the binding of TMA-DPH to different lipophorin subspecies can be described by the same lipid−water partition equilibrium, $K_p^{\text{lipid/water}}$, it is possible to estimate the relative sizes of the lipid surfaces of different lipoprotein particles. Because the size of the lipid surface, or number of surface lipid molecules, is not known, we can only determine the value of the "apparent partition constant", $K_p^{\text{Lp/water}}$, which is defined as

$$K_p^{\text{Lp/water}} = (\text{moles of probe bound/moles of lipoprotein})/(\text{moles of probe free/moles of water})$$

The relationship between the constant, $K_p^{\text{lipid/water}}$, and the apparent constant, $K_p^{\text{Lp/water}}$, is given by the following equation:

$$K_p^{\text{lipid/water}} A^{\text{lipid}} = K_p^{\text{Lp/water}} a_L$$

where A^{lipid} is the lipid surface area per mole of lipoprotein, a_L is the average molecular area of the surface lipids, and $K_p^{\text{lipid/water}}$ is given by

$$K_p^{\text{lipid/water}} = (\text{moles of probe bound/moles of surface lipid})/(\text{moles of probe free/moles of water})$$

Thus, when the binding data are represented as a function of the molar concentration of lipoprotein particles, the value of the apparent partition constant, K_p^{Lp}, is directly proportional to the size of the lipoprotein lipid surface.

The binding curves for five adult lipophorin subspecies are shown in Figure 3A. The values of the apparent partition constants, K_p^{Lp}, obtained with lipophorin subspecies isolated from the hemolymph of adult and larvae insects are shown in Figure 3B. It can be seen that, for the six lipophorin subspecies that are in the 1.09−1.17 g/mL density range, four from larvae and two HDLps from adults, the decrease

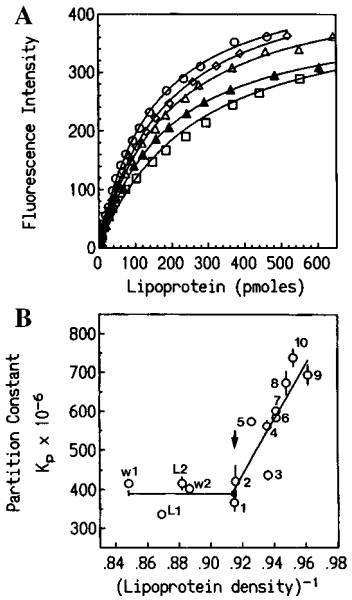

FIGURE 3: Dependency of the apparent partition constant of TMA-DPH, K_p^{Lp}, on the lipoprotein density. (A) Fluorescence enhancement curves of the adult lipophorins: HDLp, $d = 1.093$ g/mL (□); IDLp, $d = 1.074$ g/mL (▲); IDLp, $d = 1.062$ g/mL (△); IDLp, $d = 1.055$ g/mL (◇); and LDLp, $d < 1.04$ g/mL (○). (B) K_p^{Lp} values obtained from the fluorescence enhancement curves are plotted against the reciprocal of the lipoprotein density. W1 and W2 refer to lipophorin particles of densities 1.179 and 1.115 g/mL, respectively, which were obtained from insects in the prepupal stage. L1 ($d = 1.151$ g/mL) and L2 ($d = 1.134$ g/mL) were obtained from feeding fifth instar larvae. The arrow indicates the point corresponding to adult HDLp. The densities of the lipoproteins 1−10, which were obtained from adult insects, are indicated in the legend to the following figure. Error bars indicate the root mean square error.

in lipophorin density does not significantly affect the size of the lipid surface. However, for lipoprotein particles with densities lower than 1.09 g/mL, there is a clear correlation between the lipoprotein density and the partition constant. The increase in the apparent partition constant observed in the 1.04−1.09 g/mL density range indicates that loading of the lipoproteins with DG increases the size of the surface lipid phase accessible to the probe. It is important to note that loading of the lipoproteins with DG also produces an increase in the content of the surface exchangeable apolipoprotein, apoLp-III. Therefore, the increase in the apparent partition constant of TMA-DPH that accompanies the increase in DG content of lipophorins from adult insects suggests that the binding of apoLp-III which follows the loading of lipophorin with DG does not completely prevent DG molecules from reaching the lipoprotein surface. The K_p^{Lp} values of adult lipophorins indicate that during the transformation of HDLp into LDLp the size of the lipid surface increases up to 1.7-fold (Figure 3B).

The increased binding of TMA-DPH to lipoproteins of lower density could be in part due to the electrostatic interaction of the increasingly apoLp-III-enriched lipoproteins with the positively charged probe. In order to test the possible effect of such an electrostatic interaction, we measured the partition of [^{3}H]oleic acid between HDLp and LDLp by incubating a mixture of these two lipoproteins with traces of radioactive oleic acid. The lipoprotein mixture was then subjected to ultracentrifugation, and the radioactivity and protein content of the HDLp and LDLp fractions were determined. The results showed that LDLp binds 2.2-fold

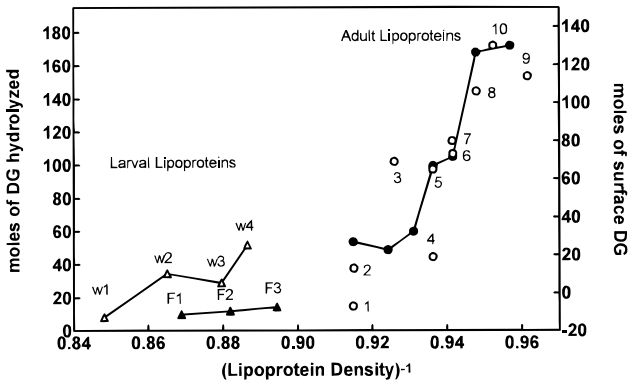

FIGURE 4: Accessible DG content. K_p-derived estimations of the surface DG content of adult insect lipoproteins, expressed in mols of DG per mole of lipoprotein, are plotted on the right axis (○): 1 and 2, HDLp-A, $d = 1.093$; 3 and 4, IDLp-A, $d = 1.068$; 5, IDLp-A, $d = 1.080$; 6 and 7, IDLp-A, $d = 1.062$; 8, IDLp-A, $d = 1.055$; 9, LDLp-A, $d \leq 1.04$; and 10, IDLp-A, $d = 1.050$ g/mL . The end point hydrolysis data are represented on the left axis for lipophorin subspecies obtained from fifth instar larvae in its feeding stage (▲), larvae in its prepupal stage (△), and adult insects (●). Densities are in grams per cubic centimeter: F**1**, $d = 1.151$; F**2**, $d = 1.134$; F**3**, $d = 1.151$; W1, $d = 1.179$; W2, $d = 1.156$; W3, $d = 1.137$; and W4, $d = 1.128$. Data are expressed in number of moles of hydrolyzed DG per mole of lipoprotein.

more oleic acid than HDLp. The similarity between the surface size increases obtained by TMA-DPH and oleic acid binding indicates that the electrostatic effect of apoLp-III on TMA-DPH binding is probably negligible.

Estimation of the Surface DG Content of Lipophorin. In order to translate the K_p^{Lp} values into the number of DG molecules that reside on the lipophorin surface, we can make use of the K_p^{Lp} values obtained with larval lipoproteins, for which the size of the lipoprotein lipid surface has been estimated. Because larval lipophorins appear to have a very low surface DG content (Soulages et al., 1994), and do not contain apoLp-III, the area of the lipoprotein lipid surface, A_{ls}, is given by $A_{ls} = N_{pl}a_{pl}$, where N_{pl} and a_{pl} are the number of phospholipid molecules and the molecular area of the phospholipid, respectively. On the other hand, K_p^{Lp} is proportional to $N_{pl}/K_p^{Lp/water} = K_p^{Lp/water}N_{pl}$. An average value for the partition constant, $K_p^{lipid/water}$, of $2.71 \times 10^6 \pm 0.45 \times 10^6$ was obtained from the PL content and K_p^{Lp} values of the four larval lipoproteins, which do not contain apoLp-III. This K_p value was employed to estimate, from the K_p^{Lp} values, the "equivalent" number of PL molecules residing on the surface of the different adult lipophorin subspecies. The estimated PL content was then compared to the actual PL content of adult lipophorin subspecies, which is about 142 molecules of PL per lipoprotein particle (Wells et al., 1987). Assuming that DG and PL have a similar molecular area of about 75 Å^2, the difference between the experimental and estimated values of N_{PL} gives an estimation of the number of DG molecules residing in the surface lipid phase of the lipoprotein. Figure 4 shows a plot of the K_p-derived surface DG contents of several adult lipophorin subspecies. These data clearly show that loading of HDLp with DG results in a progressive increase in the amount of surface DG. The ratio of surface DG content in LDLp and HDLp suggests about a 7-fold increase in the surface DG content of the lipoprotein.

In order to obtain an independent estimation of the accessible DG content of lipophorin particles, we determined the maximum extent of DG hydrolysis that can be reached when different lipophorin subspecies are subjected to the action of a TG lipase. The results, obtained with 14 lipophorin subspecies, which mostly differed in their total DG content, are shown in Figure 4 in conjunction with the K_p-derived estimations of the surface DG content. As a general trend, we observed an increase in the accessible DG content as the density of the lipoprotein decreased. The lipoproteins with the lowest susceptibility to TG lipase were those obtained from larvae in their feeding stage, where less than 15 molecules of DG were hydrolyzed in each of the three lipoprotein subspecies studied. The greatest susceptibility to TG lipase was observed in the 1.09−1.04 g/mL density range. About 50 molecules of DG were hydrolyzed in HDLp ($\rho = 1.09$ g/mL), and 170 molecules of DG were hydrolyzed in LDLp ($\rho < 1.04$ g/mL). In this density range, estimations derived from both K_p and lipase data gave a similar pattern of increase in the accessible surface DG content, as the total DG content of the lipoproteins increased. The lipase study suggested a higher accessible DG content and a smaller increase between HDLp and LDLp than the TMA-DPH partition study. However, because the treatment of lipophorin with lipase could promote the release of apoLp-III (Kawooya et al., 1991), an overestimation of the surface DG content was expected with this technique. An additional source of overestimation using the TG lipase treatment is given by the possibility that, after the hydrolysis of DG, the lipoprotein surface would be replenished with DG from the lipoprotein core. Overall, both estimations indicate that the transformation of HDLp into LDLp results in a 4−7-fold increase in the concentration of surface DG.

The feasibility of the experimental results was tested further by calculation of the surface DG content of HDLp and LDLp, making use of an extended version of the density composition model previously developed for lipophorin particles without apoLp-III (Soulages & Wells, 1994a; Soulages & Brenner, 1991). The total surface area was obtained from the diameters of the particles, which were calculated from the particle density and composition (HDLp, 65 Å^2, and LDLp, 87.1 Å^2; Soulages & Wells, 1994a), and should be equal to the sum of the areas occupied by apoLp-I, apoLp-II, apoLp-III, PL, and DG. The area occupied by apoLp-I plus apoLp-II was previously estimated to be 32 000 Å^2 (Soulages & Wells, 1994a). The area covered by PL was obtained from the PL content assuming a molecular area of 75 Å^2, and the area covered by apoLp-III was estimated from the apoLp-III content of HDLp and LDLp employing a molecular area of 2300 Å^2, as determined by adsorption of apoLp-III to PL and DG monolayers (Demel et al., 1992). From these data and assuming a molecular area of 75 Å^2 for DG, it was calculated that the number of surface DG molecules increases from 78 to 212 when HDLp is transformed into LDLp.

A comparison of the results obtained with the different methods is presented in Table 1.

Packing of the Surface Lipids. The aim of this part of the study was to compare the molecular packing of the surface lipid layer of the different lipophorin subspecies. Steady-state fluorescence anisotropy of lipid-embedded probes provides information about the average motional freedom of the lipids surrounding the fluorophore (Kawato et al., 1977). In order to measure the packing of the lipids

Table 1: Comparison of the Lipophorin Lipid Surface Sizes and Surface DG Contents Obtained by Different Methods

method	accessible or surface DG (molecules per particle)		lipid surface size (LDLp/ HDLp)	surface DG content (LDLp/HDLp)
	HDLp	LDLp		
TMA-DPH K_p-derived	17	120	1.7	7
oleic acid partition[a]	17	212	2.2	12
triacylglycerol lipase	50	170	1.6	3.4
surface space-filling model	78	212	1.6	2.7

[a] The number of molecules of surface DG in HDLp particles was assumed to be the same as that calculated in the TMA-DPH study. The surface DG content of LDLp particles was obtained from the LDLp to HDLp lipid surface sizes ratio using the same rationale employed to analyze the TMA-DPH binding data. The calculations involved in all the other methods were explained in text.

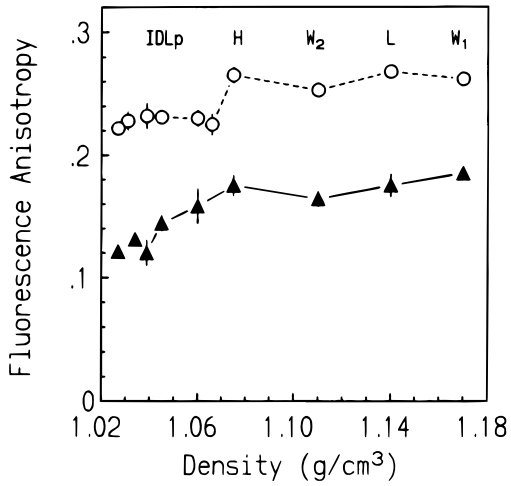

FIGURE 5: Lipid order of the lipoprotein surface. Dependency of the fluorescence anisotropy of TMA-DPH (○) and cis-PnA (▲) on the reciprocal of the lipoprotein density. $W_1$and W_2 refer to lipophorin particles isolated from insects in their prepupal-1 and 2-stages and had densities of 1.179 and 1.115 g/mL, respectively. L refers to lipophorin isolated from larvae during the feeding stage (d = 1.14 g/mL); H and IDLp refer to high-density and intermediate-density lipophorins isolated from adult insects. Error bars represent the experimental standard deviations, which range between 3×10^{-3} and 7×10^{-3}.

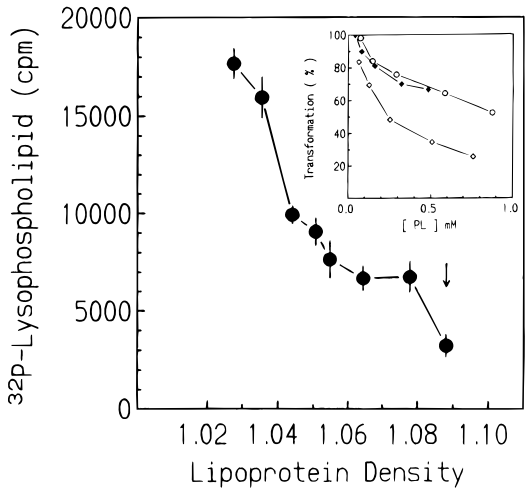

FIGURE 6: Effect of the DG content on the susceptibility of lipophorin to phospholipase A_2. The main plot shows the initial rates of PLA_2 activity against the lipoprotein density. Reactions were performed at 37 °C employing 0.15 unit of PLA_2 and 2 mM lipophorin phospholipid. The inset shows the percentage of hydrolysis of the lipophorin phospholipid in the presence of 10 units of enzyme: HDLp, d = 1.09 g/mL (◇); IDLp, d = 1.06 g/mL (◆); and LDLp, d < 1.035 g/mL (○). Error bars represent the experimental standard deviation (n = 3).

which reside in the lipophorin surface, we determined the anisotropy of fluorescence of TMA-DPH and cis-PnA. These two fluorophores are located in the lipoprotein surface (Ben-Yashar & Barenholz, 1991). Figure 5 shows the anisotropy of fluorescence of TMA-DPH and cis-PnA embedded in the surface of 10 different lipophorin subspecies. The results obtained with both fluorophores indicate that lipophorin particles containing apoLp-III have a less packed lipid surface than lipophorins of densities higher than 1.10 g/mL, which do not contain apoLp-III. Among the adult lipophorin particles, which contain apoLp-III and are in the 1.09−1.04 g/mL density range, small differences in the packing of the surface lipid were detected by TMA-DPH. However, the data obtained with cis-PnA indicate that the increase in the DG content of lipophorin is accompanied by a small but progressive decrease in the surface lipid packing.

Susceptibility to Phospholipase A2. The susceptibility of lipophorin phospholipids to hydrolysis by PLA_2 was employed as a probe of the effect of the increasing DG content on the physicochemical properties of the lipophorin surface. Lipophorin particles of different density were radiolabeled in their phospholipid moiety with ^{32}P and employed as PLA_2 substrates. Twenty-four hours after the injection of $^{32}PO_4H_3$, the lipophorin particles of different density contained the same specific activity in their PL moiety. Chemical and NMR analyses have indicated that PE and PC represent about 35 and 65% of the total phospholipid content of *M. sexta* lipophorin, respectively (Wang et al., 1992). We found that PE and PC contained 47 and 44% of the PL counts per minute, respectively. The remaining 10% of the radioactivity was distributed between lysophospholipids, 6%, and other unidentified lipids. The apparent higher labeling of PE might indicate that the turnover rates of PE and PC are different. Given the fact that all the lipophorin subspecies from adult insects have the same phospholipid content and composition, and all were radiolabeled to the same extent, the comparison of the PLA_2 activity among different lipophorin subspecies was straightforward. In the presence of excess of PLA_2, IDLp (d = 1.06 g/mL) and LDLp (d < 1.03 g/mL) were much more susceptible to hydrolysis that HDLp. However, no differences were observed among IDLp subspecies, including LDLp (Figure 6A). PE was more susceptible to hydrolysis than PC, and the average degree of hydrolysis of PE was 1.3 times that of PC.

When the concentration of enzyme was decreased to measure initial velocities, the activity of the enzyme was shown to be dependent on the DG content of the lipophorin. As shown in Figure 6B, the increase in the lipophorin DG content resulted in an increased susceptibility of the lipophorin phospholipid to PLA_2. For example, LDLp (d < 1.03 g/mL) showed a 5-fold higher rate of hydrolysis than HDLp (d = 1.088 g/mL).

DISCUSSION

Lipophorin acts as a reusable shuttle transporting lipids, mainly DG, from the sites of synthesis and absorption to the sites of storage and utilization (Chino & Kitazawa, 1981; Downer & Chino, 1985). Several factors can modify the metabolism of hemolymph DG. For example, adipokinetic hormone (AKH), which appears to control the level of hemolymph lipid during flight, increases the concentration of DG in the hemolymph (Beenakkers et al., 1985), which translates into an increase in the concentration of low-density lipoprotein particles with a high DG content. Because lipid mobilization originated from an increased energy demand, it follows that the rate of utilization of fatty acids should be increased. However, an increased hemolymph DG concentration does not ensure, by itself, a more rapid metabolism of fatty acids because the hemolymph DG must be hydrolyzed before the fatty acids can be utilized by tissues. The alterations in the physicochemical properties of the lipoprotein surface which result from an increased DG content can constitute a major factor that controls the rate of utilization and metabolism of the DG by affecting the interactions of the lipoprotein with enzymes and receptors.

Composition of the Lipophorin Surface. Phospholipids are located in the lipoprotein surface (Katagiri, 1985), whereas the surface concentration of DG appears to be very low in lipophorin particles that do not contain apoLp-III (Soulages & Brenner, 1991; Soulages & Wells, 1994a; Soulages et al., 1994). Several studies indicated that the accumulation of free DG on the lipophorin surface would not be compatible with the stability of the particles. *In vitro* loading of lipophorin with DG in the absence of apoLp-III produces unstable particles that aggregate, although stable particles are formed when the loading takes place in the presence of apoLp-III (Soulages & Wells, 1994b). In a lipid membrane model system, we have recently shown that very low concentrations of DG promoted the binding of apoLp-III (Soulages et al., 1995). It has also been shown that extensive hydrolysis of the lipophorin PL to DG by phospholipase C promotes binding of apoLp-III to the lipophorin particle or to lipoprotein aggregation in the absence of apoLp-III (Singh et al., 1994). In the context of these previous studies, it would have been reasonable to assume that the concentration of free DG on the surface of lipophorin particles containing apoLp-III would be low.

In a recent report, Wang et al. (1995) presented experimental evidence supporting the existence of a surface DG pool in *M. sexta* LDLp. However, neither the nature nor the quantitation of the surface DG pool in lower-density lipophorins has been previously reported. Our studies suggest that, in spite of the binding of apoLp-III, loading of lipophorin with DG results in an increase in the size and DG content of the lipophorin lipid surface. The increase in the number of amphipathic ligand binding sites and the end point hydrolysis study indicate that the formation of LDLp is accompanied by a 4−7-fold increase in the surface content of DG. The presence of a considerable number of DG molecules on the surface of stable lipophorin particles might be possible due to the electrostatic barrier caused by the negatively charged apoLp-III molecules. In addition, the binding of apoLp-III would maintain a high lateral pressure in the surface, which would minimize the formation of packing defects on the lipoprotein surface as a consequence of the presence of DG.

Properties of the Lipophorin Surface. The high sensitivity of PLA_2 from porcine pancreas to many of the physicochemical factors that can affect the surface properties of its substrate (Volwerk et al., 1986; Jain & Zakim, 1987) makes this enzyme a useful tool in probing how DG loading changes the properties of the PL in the surface. It has also been shown in different systems that *sn*-1,2-DG stimulates the degradation of PC by PLA_2 (Dawson et al., 1983; Kolesnic & Paley, 1987; Cunningham et al., 1989). In addition to the known properties of PLA_2, the choice of this enzyme as a probe of the lipophorin surface lipid phase was based on the fact that lipophorin subspecies have the property that they contain a constant number of phospholipid molecules per particle, which greatly facilitates the interpretation of the data. Given the apparent increase in the size of the lipophorin lipid surface that accompanies the loading of the lipoprotein with DG, it would have been reasonable to expect a lower PLA_2 activity against LDLp due to a decrease in the surface concentration of PL produced by the incorporation of DG and apoLp-III into the lipoprotein surface. The observed increase in enzyme activity which accompanies the decrease in lipophorin density suggests that the increase in surface DG content causes considerable perturbation of the surface, which makes the PL of the lipoprotein more susceptible to hydrolytic cleavage, despite the dilution of surface PL. This interpretation would be consistent with the observation that incorporation of DG into PC liposomes increases the activity of PLA_2 against such liposomes (Kolesnic & Paley, 1987; Cunningham et al., 1989). It is also noteworthy that there is a striking similarity found between the pattern of enzyme activity and the estimations of surface DG content of lipophorin. Other factors, such as the reduction of steric hindrance for the interaction of PLA_2 with the PL in the surface that should accompany the increase in the lipoprotein lipid surface size or the perturbation generated by the increased content of apoLp-III and/or DG that accompanies the transformation of HDLp into LDLp, could also be involved. It is also possible that the higher fluidity of the lipid surface of IDLps and LDLp could also be involved in the modulation of the PLA_2 activity. Although the nature of the surface properties of the lipoprotein that promote the activation of PLA_2 cannot be unequivocally identified, the observed activation is relevant because it clearly shows that loading of lipophorin with DG and apoLp-III can effectively promote a large variation in enzyme−lipoprotein lipid interaction.

Metabolic Implications of the Properties of the Lipophorin Surface. The increase in the surface DG content of lipophorin that accompanies the formation of IDLp and LDLp is in agreement with the physiological role of these particles, which is to deliver large amounts of fatty acids to the flight muscle. Moreover, the concentration of surface DG in LDLp and HDLp could explain the higher activity of *M. sexta* lipoprotein lipase against LDLp-DG (Van Heusden, 1993). The increase in the accessible DG content is also consistent with an *in vivo* study, which showed that the turnover rate of LDLp−DG in *Acherontia atropos* during flight is 2−5 times faster than the turnover rate of HDLp−DG in resting insects (Surtholt et al., 1991). In addition to the increase in hemolymph DG during the insect flight, a large increase in the concentration of hemolymph free fatty acids has also been

observed. A 6-fold increase was observed in *Locusta migratoria* (Beenakkers et al., 1985), and a 3-fold increase was found in *M. sexta* (J. L. Soulages, unpublished data). It has been previously shown that lipophorin is the major carrier of hemolymph FFA in adult insects (Soulages & Wells, 1994c; Gonzalez et al., 1991). The increase in the number of binding sites for amphipathic compounds that accompanies the increase in the size of the lipoprotein lipid surface is consistent with the role of lipophorin in the transport of FFA. The larger lipid surface size of LDLp thus allows the solubilization and transport of a high concentration of hemolymph FFA (up to 1.2 mg/mL), which otherwise could damage cells and tissues.

Summary and Conclusions. Loading of lipophorin particles with DG and apoLp-III generates an increase in the size of the accessible surface lipid phase. This increase is the result of a larger number of DG molecules residing in the lipoprotein surface. Both the 2-fold increase in the size of the lipid surface and the 4−7-fold increase in the number of surface DG molecules are predicted to facilitate the degradation of DG. Also, the increase in the size of the lipoprotein lipid surface that follows the transformation of HDLp into LDLp allows the solubilization and transport of a higher FFA concentration. The increase in the size of the surface lipid pool might interact synergistically with the changes in the electrostatic surface properties generated by the binding of apoLp-III to increase the metabolic reactivity of DG-loaded lipophorins. We propose that the physicochemical modification of the lipophorin surface resulting from DG loading enhances the interaction of the lipoprotein with enzymes and receptors leading to a faster rate of DG degradation and fatty acid utilization. We suggest that this mechanism could be a major factor in controlling the rate of DG hydrolysis.

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REFERENCES

Azumi, T., & McGlyn, S. P. (1962) *J. Chem. Phys. 37*, 2413−2420.
Beenakkers, A. M. Th., Van der Horst, D. J., & Van Marrewijt, W. J. A. (1984) *Insect Biochem. 14*, 243−260.
Beenakkers, A. M. Th., Van der Horst, D. J., & Van Marrewijk, W. J. A. (1985) *Prog. Lipid Res. 24*, 19−67.
Ben-Yashar, V., & Barenholz, Y. (1991) *Chem. Phys. Lipids 60*, 1−14.
Blacklock, B. J., & Ryan, R. O. (1994) *Insect Biochem. Mol. Biol. 24*, 855−873.
Chino, C., & Kitazawa, K. (1981) *J. Lipid Res. 22*, 1042−1052.
Cunningham, B. A., Tsujita, T., & Brockman, H. L. (1989) *Biochemistry 28*, 32−40.
Dawson, R. M. C., Hemington, N. L., & Irvine, R. F. (1983) *Biochem. Biophys. Res. Commun. 117*, 196−201.
Demel, R. A., van Doorn, J. M., & van der Horst, D. J. (1992) *Biochim. Biophys. Acta 1124*, 151−158.
Downer, R. G. H., & Chino, H. (l985) *Insect Biochem. 15*, 627−630.
Gonzalez, M. S., Soulages, J. L., &Brenner, R. R. (1991). *Insect Biochem. 21*, 679−687.
Jain, M. K., & Zakim, D. (1987) *Biochim. Biophys. Acta 906*, 33−68.
Katagiri, C. (1985) *Biochim. Biophys. Acta 834*, 139−143.
Kawato, S., Kinosita, K., & Ikegami, A. (1977) *Biochemistry 16*, 2319−2324.
Kawooya, J. K., Van der Horst, D., Van Heusden, M. C., Brigot, B. L., Van Antwerpen, R., & Law, J. H. (1991) *J. Lipid Res. 32*, 1781−1788.
Kolesnick, R. N., & Paley, A. E. (1987) *J. Biol. Chem. 262*, 9204−9210.
Nagao, E., & Chino, H. (1991) *J. Lipid Res. 32*, 417−422.
Nichols, A. V., Krauss, M. R., & Musliner, T. A. (1986) *Methods in Enzymol. 128*, 417−431.
Prasad, S. V., Ryan, R. O., Law, J. H., & Wells, M. A. (1986) *J. Biol. Chem. 261*, 558−562.
Shapiro, J. P., Keim, P. S., & Law, J. H. (1984) *J. Biol. Chem. 259*, 3680−3685.
Shapiro, J. P., Law, J. H., & Wells, M. A. (1988) *Annu. Rev. Entomol. 33*, 297−318.
Singh, T. K. A., Liu, H., Bradley, R., Scraba, D. G., & Ryan, R. O. (1994) *J. Lipid Res. 35*, 1561−1569.
Soulages, J. L., & Brenner, R. R. (1991) *J. Lipid Res. 32*, 407−415.
Soulages, J. L., & Wells, M. A. (1994a) *Adv. Protein Chem. 45*, 371−415.
Soulages, J. L., & Wells, M. A. (1994b) *Biochemistry 33*, 2356−2362.
Soulages, J. L., & Wells, M. A. (1994c) *Insect Biochem. 24*, 79−86.
Soulages, J. L., Rimoldi, O. J., & Brenner, R. R. (1988) *J. Lipid Res. 29*, 172−182.
Soulages, J. L., Rivera, M. F., Walker, F. A., & Wells, M. A. (1994) *Biochemistry 33*, 3245−3251.
Soulages, J. L., Salamon, Z., Wells, M. A., & Tollin, G. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5650−5654.
Sparks, D. L., & Phillips, M. C. (1992) *J. Lipid Res. 33*, 123−130.
Surtholt, B., Goldberg, J., Schulz, T. K. F., Beenakkers, Ad. M. Th., & Van der Horst, D. J. (1991) *Biochim. Biophys. Acta 1086*, 15−21.
Van der Horst, D. J. (1990) *Biochim. Biophys. Acta 1047*, 195−211.
Van Heusden, M. C. (1993) *Insect Biochem. Mol. Biol. 23*, 785−792.
Volwerk, J. J., Jost, P. C., de Hass, G. H., & Griffith, O. H. (1986) *Biochemistry 25*, 1726−1733.
Wang, J., Liu, H., Sykes, B. D., & Ryan, R. O. (1992) *Biochemistry 31*, 8706−8712.
Wang, J., Liu, H., Sykes, B. D., & Ryan, R. O. (1995) *Biochemistry 34*, 6755−6761
Wells, M. A., Ryan, R. O., Kawooya, J. K., & Law, J. H. (1987) *J. Biol. Chem. 262*, 4172−4176.

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