^{31}P-NMR Study of the Phospholipid Moiety of Lipophorin Subspecies†

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ABSTRACT: ^{31}P-NMR spectra of four distinct subspecies of *Manduca sexta* hemolymph lipophorin revealed the presence of two resonances separated by 0.6 ppm. Phospholipid analysis of the lipoproteins showed that phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were present and their mass ratio correlated well to the intensity of the two resonances in each of the different subspecies. The two resonances persisted in ^{31}P-NMR spectra of organic solvent extracts of lipophorin. These results, together with the fact that PE, but not PC, can form an intramolecular hydrogen bond between the phosphate oxygen and the amino group of ethanolamine, resulting in deshielding of the phosphorus nucleus (and a 0.6 ppm downfield shift), strongly suggest the resonances observed represent the PC and PE components of these lipoproteins. ^{31}P-NMR line-width data obtained as a function of temperature and solvent viscosity were used to calculate the chemical shift anisotropy ($\Delta\sigma$), intrinsic viscosity (η'), and lateral diffusion coefficients (D_T) of PC and PE in different lipophorin subspecies. η' and D_T for PC and PE were similar among high-density lipophorins but differed in low-density lipophorin (LDLp). These differences may be related to the large increase in diacylglycerol content in this particle and/or the association of up to 16 molecules of apolipophorin III. On the basis of the known lipid compositional differences between LDLp and high-density lipophorin subspecies, we propose that uptake of large amounts of diacylglycerol during LDLp formation results in partitioning of this lipid to the surface monolayer where it intercalates between phospholipid molecules. Diacylglycerol intercalation creates gaps between phospholipid head groups that expose the hydrophobic surface. This surface is stabilized by apolipophorin III binding. Together these modifications in the surface monolayer are reflected in the phospholipid ^{31}P-NMR line widths, η', and D_T of this lipoprotein.

In insect hemolymph hydrophobic biomolecules are transported via lipid–protein complexes termed lipophorins (Shapiro et al., 1988; Ryan, 1990). The properties of lipophorin have been studied in detail, and considerable information is available on their size, apolipoprotein content, and lipid composition. In the tobacco hornworm *Manduca sexta*, it has been shown that, during development or in response to hormonal stimuli, dramatic alterations occur in the lipid content and composition of lipophorin particles. Since many of these alterations occur by remodeling preexisting lipophorin particles, it has been hypothesized that there exists a basic apolipoprotein–phospholipid matrix structure that is capable of accepting or donating lipid to form distinct subspecies. In spite of large variations in particle lipid content, all lipophorin particles possess one molecule each of the integral apolipoprotein components, apolipophorin I and apolipophorin II (Shapiro et al., 1984). This structural resiliency is a cornerstone of the reusable shuttle hypothesis of lipophorin function first proposed by Chino (Chino & Kitazawa, 1981; Chino, 1985).

In contrast to mammalian lipoproteins, diacylglycerol (DAG)[1] is the transport form of neutral lipid by lipophorin. Lipophorin subspecies exist which range in DAG content from 4% to >20% of the particle mass (Kawooya et al., 1988; Prasad et al., 1986). Further increases in DAG content are observed when a third, low molecular weight, water-soluble apoprotein, apolipophorin III (apoLp-III), associates with the particle (Kawooya et al., 1984; Wells et al., 1987; Brieter et al., 1991). High-density lipophorin adult (HDLp-A) contains 25% DAG and two nonexchangeable apoLp-III, while low-density lipophorin (LDLp) contains up to 16 molecules of apoLp-III and has a DAG content of 46% by weight (Ryan et al., 1986; Wells et al., 1987). The significance of these differences in DAG content with respect to the structure or function of lipophorins has not been elucidated. It has been hypothesized that, owing to its relative polarity, DAG can partition between the surface and the core of lipophorin particles and, as such, be accessible to transfer from the particle (Ryan et al., 1988).

The other major lipid component of lipophorin is phospholipid which comprises between 14% and 25% of the mass of different high-density lipophorin (HDLp) subspecies (Shapiro et al., 1988). In ^{31}P-NMR and phospholipase A_2 digestion studies, Katagiri (1985) demonstrated that the phospholipid component of *Locusta migratoria* lipophorin is localized in a monolayer on the surface of the particle. This observation is consistent with studies on the location of mammalian lipoprotein phospholipid at the surface of those particles (Keim, 1979). In the present study we have used ^{31}P-NMR spectroscopy to investigate four distinct subspecies of *M. sexta* lipophorin, and on the basis of our analysis, the implications of our results with respect to the structure of lipophorin are discussed.

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[1] Abbreviations: NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; Lp, lipophorin; apoLp, apolipophorin; HDLp-W2, high-density lipophorin wanderer 2; HDLp-W1, high-density lipophorin wanderer 1; HDLp-A, high-density lipophorin adult; LDLp, low-density lipophorin; DAG, diacylglycerol.

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MATERIALS AND METHODS

Preparation of Lipophorins. Lipophorins were isolated from freshly collected hemolymph of larval or adult stage *M. sexta* by density gradient ultracentrifugation (Shapiro et al., 1984). HDLp-W1 and HDLp-W2 were isolated from prepupal larvae as described by Prasad et al. (1986). HDLp-A and LDLp were isolated from 1-day-old adult moths according to Ryan et al. (1986). In some cases, lipophorin samples were concentrated by KBr flotation ultracentrifugation at 1.21 g/mL in a Ti 50.2 rotor at 40 000 rpm for 16 h. The purity of isolated lipophorins was assessed by SDS–PAGE.

Lipid Analysis. Different lipophorin subspecies were extracted with chloroform/methanol according to Bligh and Dyer (1959). Extracts were dried under a stream of N_2, and lipids were separated by thin-layer chromatography on glass plates, precoated with silica gel 60, in a solvent system composed of chloroform/methanol/acetic acid/H_2O (50/30/8/3 v/v/v/v). After separation, the bands corresponding to phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin were scraped from the plate and subjected to phosphorus analysis (Rouser et al., 1966).

NMR Sample Preparation. Prior to NMR, lipophorins were dialyzed into 30 mM PIPES, 50 mM NaCl, and 1 mM Na_2EDTA (pH. 6.8) for 16 h. After dialysis, the samples were used directly for NMR measurements. Using a 10-mm NMR tube, 2.5 mL of the lipophorin sample was mixed with 0.5 mL of D_2O at 4 °C and placed in the NMR tube. Twenty-five microliters of 50 mM inorganic phosphate buffer was added as a reference standard. With the exception of LDLp, lipophorin samples were stable for up to 2 weeks at 4 °C and gave identical ^{31}P-NMR spectra after exposure to 45 °C for several hours. LDLp samples were less stable and thus were used for experiments within 4 days of isolation.

NMR Methods. ^{31}P-NMR experiments were performed on a Varian Unity 300 NMR spectrometer at 121.4 MHz without proton decoupling. The instrument was equipped with a 300 MHz wide bore magnet for 10-mm NMR tubes. All spectra were taken by using a 90° pulse with a 2.0-s acquisition time and a relaxation delay of 5–10 s (approximately 5 times T_1). Samples were equilibrated for at least 30 min at a given temperature before data were acquired. Spectra were obtained over the range of 0–39 °C with 64–128 transients. The spectral width was 2000 Hz. All spectra were processed by using the line-broadening parameter equal to 0.5 Hz.

Spectra of lipophorin subspecies at higher temperatures, such as 25 °C, showed two well-separated peaks. As described below, the downfield peak was assigned to PE and the upfield peak to PC. At low temperatures these two peaks were not well resolved. In order to obtain the true line width of the peaks at all temperatures, an iterative five-parameter curve fit program (chemical shift of PE, chemical shift of PC, line width of PE, line width of PC, and ratio of intensity of two peaks) was used to fit both PE and PC resonances to double-Lorentzian functions. During the curve fit procedure, the intensity ratio of the two peaks, derived from the intensity ratio of the two peaks in different subspecies at 39 °C, was fixed. As described below, the intensity ratio of the two peaks at 39 °C for each lipophorin was in good agreement the PE/PC ratio of the same lipophorin determined by the other methods.

Calculations. The results obtained for intrinsic viscosity, η', and chemical shift anisotropy, $\Delta\sigma$, were based on theoretical considerations (see below) and by using SigmaPlot, version 4.0 (Jandel Scientific, Corte Madera, CA), to curve fit our experimental data. During the curve fit procedure, lipophorin particle radii, determined by electron microscopy (Ryan et al., 1990, 1992), were fixed. Constraints were also used for intrinsic viscosity (0–10 P; Edidin, 1974; Marsh, 1988) and chemical shift anisotropy (1–1400 ppm; Fenske et al., 1990).

THEORY

The isotropic motion of the head group of lipoprotein phospholipids causes ^{31}P-NMR line broadening. This motion arises from two different contributions: whole particle tumbling and phospholipid lateral diffusion within the surface monolayer. Lateral diffusion is a local motion and is much faster than lipoprotein particle tumbling. These two motions modulate ^{31}P-NMR line broadening. The ^{31}P-NMR line width of phospholipids in lipoproteins can be written as (Abragam, 1961; Mclaughlin et al., 1975; Fenske et al., 1990)

$$1/T_2 = M_2\tau_c + C \tag{1}$$

where M_2 is the residual second moment obtained after averaging due to the restricted anisotropic motion of phospholipids in the plane of the monolayer of the lipoprotein, which is given by the equation (McLaughlin et al., 1975)

$$M_2 = (4/45)(2\pi\nu_0)^2(\Delta\sigma)^2 \tag{2}$$

M_2 is a constant within the temperature range of the present study (Abragam, 1961), C is the portion of the line width which is independent of the lipoprotein particle motional correlation time, τ_c (Cullis, 1976), and ν_0 is the ^{31}P absorption frequency

$$1/\tau_c = (6/\mathbf{a}^2)D_{\text{diff}} \tag{3}$$

where $\mathbf{a}$ is the particle radius and D_{diff} is the diffusion rate which consists of two parts:

$$D_{\text{diff}} = D_{\text{tumbling}} + D_{\text{lateral}} \tag{4}$$

Diffusion arising from whole lipoprotein particle tumbling is given by the Stokes–Einstein relation:

$$D_{\text{tumbling}} = kT/8\pi\mathbf{a}\eta \tag{5}$$

where η is the viscosity of the medium. If we assume that the intrinsic viscosity, η', of the phospholipid monolayer is homogeneous for each phospholipid class and the phospholipid molecular shape can be described as cylindrical, then the lateral diffusion of phospholipid on the lipoprotein surface can be given by (Saffman & Delbrück, 1975)

$$D_{\text{lateral}} = (kT/4\pi\eta' h)[\log(\eta' h/\eta r) - \gamma] \tag{6}$$

where γ is Eular's constant and r and h are the radius and the length of the phospholipid cylinder which can be found elsewhere (Marsh, 1990).

Combining eq 1 with eq 3, we can obtain

$$1/T_2 = M_2(\mathbf{a}^2/6D_{\text{diff}}) + C \tag{7}$$

By rearrangement of eq 7 with eqs 4–6, the following equation can be derived:

$$(1/T_2 - C)^{-1} = A_1(T/\eta) + A_2T + A_3(T\log\eta) \tag{8}$$

where

$$A_1 = 3k/(4\pi \mathbf{a}^3 M_2) \quad (9)$$

$$A_2 = 3k[\log(\eta' h/r) - \gamma]/2\pi M_2 \mathbf{a}^2 \eta' h \quad (10)$$

$$A_3 = -3k/(2\pi M_2 \mathbf{a}^2 \eta' h) \quad (11)$$

Since no phospholipid phase transitions occur in lipophorins over the temperature range 0–37 °C (Katagiri et al., 1985), it is reasonable to assume that η' is independent of temperature within this range. The C term in eq 7 is complex and is attributable to instrumental parameters such as line broadening, acquisition time, and phospholipid chemical shift anisotropy resulting from phospholipid interactions with other particle components as well as overall particle structure (McLaughlin et al., 1975). For sonicated egg yolk lecithin bilayer vesicles, C is a constant equal to 15 Hz (Cullis, 1976). The same line width for phospholipid resonances within the surface monolayer of lipoproteins, however, is unknown and needs to be determined experimentally. The radius, **a**, of different lipophorins has been estimated from electron microscopic studies (Ryan et al., 1990; Kawooya et al., 1991; Ryan et al., 1992). LDLp and HDLp-A are spherical while HDLp-W1 and HDLp-W2 have axial ratios <1.4. Thus, M_2 and η' in eq 8 can be treated as parameters, which can be determined for the PC and PE components of lipophorins as a function of temperature and viscosity, by least-squares fit of eq 8. From the values of M_2 and η', information on the lipophorin phospholipid chemical microenvironment can be obtained.

RESULTS

Assignment of ^{31}P-NMR Signals. The ^{31}P-NMR spectrum of *M. sexta* HDLp-W1 at two different temperatures is shown in Figure 1. The spectrum at 39 °C is characterized by two well-resolved resonances at −2.39 and −1.79 ppm while at 3 °C these resonances are less well resolved. By contrast, the ^{31}P-NMR spectrum reported for *L. migratoria* high-density lipophorin contained only a single resonance which was attributed to PC (Katagiri, 1985). The presence of two distinct resonances in *M. sexta* HDLp-W1 could conceivably be due to the existence of a portion of the phospholipid moiety of this particle in a different microenvironment, or the two resonances may arise from different phospholipid classes. These possibilities were examined by obtaining a spectrum of a lipid extract of HDLp-W1 in $CDCl_3$ (data not shown). The spectrum gave rise to two resonances in the same proportion as that found in intact HDLp-W1, indicating that the two resonances observed are not the result of distinctive protein–phospholipid or phospholipid–core lipid interactions but rather appear to be an intrinsic feature of the phospholipid components of this lipophorin. It is known from earlier ^{31}P-NMR studies that certain phospholipid resonances, including those for PE, sphingomyelin, and phosphatidylserine are shifted downfield relative to PC due to an ability to form an intramolecular hydrogen bond between the phosphate oxygen and amino or hydroxyl groups within the molecule, which results in deshielding of the phosphorus nucleus (Henderson et al., 1974).

Since previous studies have shown that, in addition to PC, *M. sexta* larval lipophorin contains significant amounts of PE and sphingomyelin (Pattnaik et al., 1979), we determined the PC/(PE + sphingomyelin) phospholipid mass ratio in HDLp-W1 by two independent methods. As shown in Table I, the results correlate well to the integration of the two peaks observed in ^{31}P-NMR spectra of HDLp-W1. When ^{31}P-NMR spectra for other lipophorin subspecies were obtained, they invariably revealed the presence of two resonances, although their ratios were different from those present in HDLp-W1. Mass analysis of the phospholipid component of these subspecies, however, was in good agreement with the observed ratio of the two resonances in each lipophorin subspecies (Table I). Thus we conclude that, of the two resonances observed in ^{31}P-NMR spectra of *M. sexta* lipophorins, the upfield peak is due to PC while the downfield peak is due to PE (plus a small amount of sphingomyelin).

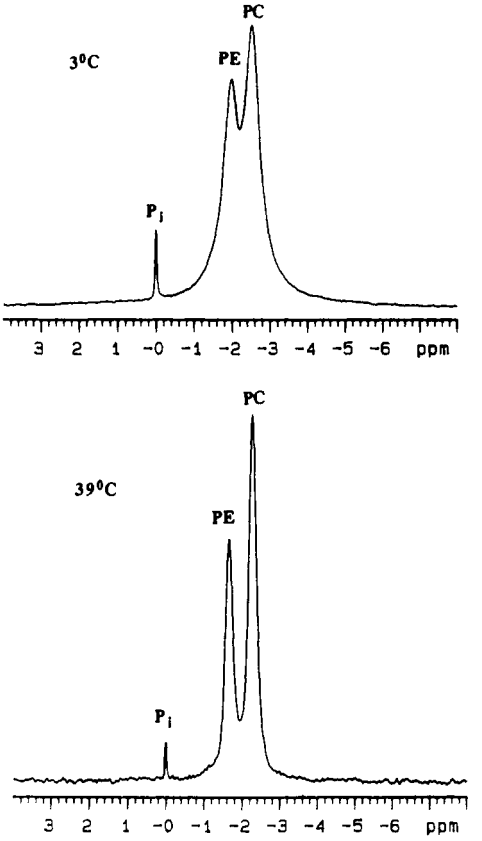

FIGURE 1: ^{31}P-NMR spectra of HDLp-W1 at 3 and 39 °C obtained on samples dialyzed against 30 mM PIPES, pH 6.8, 50 mM NaCl, and 1 mM EDTA for 16 h prior to data collection. The resonance labeled P_i represents the inorganic phosphate internal standard.

Table I: Ratio of (PE + Sphingomyelin)/PC in Lipophorin Subspecies

	(PE + sphingomyelin)/PC	
	NMR[a]	inorganic[b]
HDLp-W2	0.74	0.74
HDLp-W1	0.60	0.62
HDLp-A	0.46	0.56
LDLp	0.45	0.48

[a] Data were obtained from the integrated area of the PC and PE resonances at 39 °C. [b] Phosphorus analysis was conducted on the PC, PE, and sphingomyelin components of lipophorin extracts after separation by thin-layer chromatography according to Rouser et al. (1966). Since ^{31}P-NMR resonances for PE and sphingomyelin have the same chemical shift values, their amounts, obtained by phosphorus analysis, have been combined for comparison with the ^{31}P-NMR data.

^{31}P-NMR Line Widths of PC and PE in Different Lipophorin Subspecies. The ^{31}P-NMR line widths for PC and PE at 27 °C for each of the four lipophorin subspecies examined in this study are given in Table II. For HDLp-W2, HDLp-W1, and HDLp-A the $\Delta\nu_{1/2}$ values for PC and PE were very similar, while those for LDLp were broadened by 7–10 Hz. In each subspecies there was a constant 0.61 ± 0.01 ppm

Table II: ^{31}P-NMR Line Widths of PC and PE in *M. sexta* Lipophorins

subspecies	line width $\Delta\nu_{1/2}$ (Hz)[a]	
	PC	PE
HDLp-W2	32.1	30.3
HDLp-W1	33.3	29.8
HDLp-A	32.1	32.2
LDLp	43.5	39.6

[a] Data were collected at 27 °C in 30 mM PIPES, pH 6.8, 50 mM NaCl, and 1 mM EDTA buffer, and line widths were determined by double-Lorentzian curve fitting of the experimental data.

chemical shift difference between PC and PE resonances. In all cases plots of chemical shift versus temperature were linear over the range 0–39 °C.

Determination of the τ_c-Independent Portion of the Line Width of ^{31}P-NMR Resonances for PC and PE in Different Lipophorin Subspecies. ^{31}P-NMR spectra of HDLp-W1, HDLp-W2, HDLp-A, and LDLp were obtained at temperatures ranging from 0 to 39 °C. Solvent viscosity varied as a function of temperature between 0.5 and 2.4 cP over this temperature range. From the experimental data, we determined the ^{31}P-NMR τ_c-independent portion of the line width (C) for both PC and PE in different lipophorin subspecies from plots of $1/T_2$ vs η/T as shown in Figure 2. The calculated values of C are given in Table III. In general, the values of C for PC and PE in all lipophorin subspecies were less than C determined by Cullis (1976) for egg PC in sonicated vesicles (15 Hz) and were close to the corresponding line width determined for PC and sphingomyelin in human low-density lipoprotein (unpublished results). The C values obtained for PE compared to PC in the same lipophorin subspecies were consistently larger. For HDLp-W2, HDLp-W1, and HDLp-A, C values for PE were about 2 times greater than those of PC, but for LDLp, the C value for PE was only slightly larger than that of PC.

Chemical Shift Anisotropies, Intrinsic Viscosities, and Lateral Diffusion Coefficients of PC and PE in Lipophorin Subspecies. From the ^{31}P-NMR line-width data obtained as a function of temperature and solvent viscosity, the chemical shift anisotropy and intrinsic viscosity of PC and PE in different lipophorin subspecies were calculated. Initial values for both parameters were selected at random, and by fitting the experimental data, the program ultimately arrived at the values reported in Table IV. The observation that, in all cases, η' values for PC and PE within the same subspecies are essentially the same suggests the surface phospholipids of lipophorins can be considered to be in a homogeneous environment. The data also show that, among HDLp-W2, HDLp-W1, and HDLp-A, intrinsic viscosities are very similiar but differ considerably from the corresponding values in LDLp, which are about 4 times greater than values for other lipophorin subspecies. The intrinsic viscosity values obtained are within the range reported for a variety of membrane systems (Ladbrooke et al., 1968; Cone, 1972; Radda & Smith, 1970; Rudy & Gitler, 1972; Grisham & Barnett, 1973). Unlike η', the $\Delta\sigma$ decreases steadily from HDLp-W2 to HDLp-W1 to HDLp-A to LDLp. No significant chemical shift anisotropy differences, however, were found between PC and PE within the same lipophorin particle. D_T values were similar for PC and PE within each subspecies and between HDLp-W2, HDLp-W1, and HDLp-A. However, D_T values for PC and PE in LDLp were approximately one-third of those observed in other subspecies. These data provide support for the concept

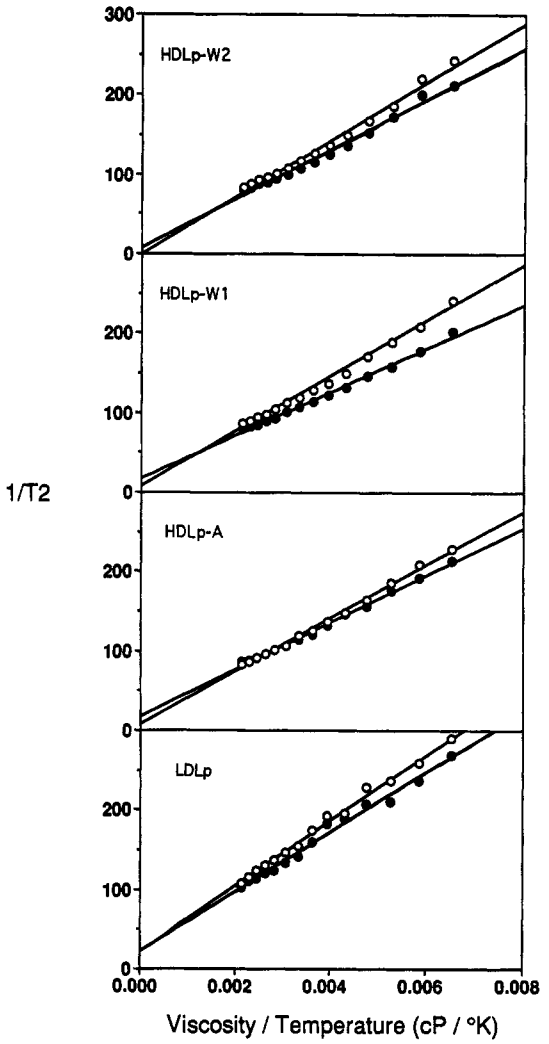

FIGURE 2: $1/T_2$ versus η/T for the ^{31}P resonances of HDLp-W2, HDLp-W1, HDLp-A, and LDLp over the temperature range 0–39 °C and viscosity range 0.5–2.4 cP. The lines shown were obtained by linear curve fitting of the data (correlation coefficient > 0.99). Open symbols correspond to PC resonances, and filled symbols correspond to PE resonances.

that the phospholipid components of LDLp exist in a more restricted environment than that in other subspecies.

DISCUSSION

^{31}P-NMR spectroscopy has been used to study the structure and dynamics of phospholipids in different subspecies of *M. sexta* lipophorin. Lipophorin particle motion independent line widths were obtained experimentally for each of four different lipophorin subspecies. *M. sexta* lipophorins are unique compared to other well-characterized lipoproteins because they possess relatively large amounts of PE. The observation that lipophorin PE and PC resonances are well resolved permitted quantitation of differences between PC and PE in the same lipophorin subspecies and among different lipophorin subspecies. The data were interpreted on the basis of structural differences between phospholipid head groups in the same lipophorin as well as possible interactions between phospholipids and lipid or protein in different lipophorin subspecies. The content of DAG as well as the surface apolipoprotein, apoLp-III, differs in these respective particles and therefore may influence the spectral properties. Toward this end, the structure and motion of PC and PE on the lipophorin particle surface have been characterized by their intrinsic viscosity,

Table III: τ_c-Independent Portion of ^{31}P-NMR Line Widths (C) of the PC and PE Components of Lipophorin Subspecies[a]

	HDLp-W2		HDLp-W1		HDLp-A		LDLp	
	PE	PC	PE	PC	PE	PC	PE	PC
C (Hz)	2.45	1.20	5.57	2.15	5.82	2.44	7.30	7.13

[a] Values were obtained from the data in Figure 2. The Y intercept of the data for each subspecies was divided by π to convert $1/T_2$ to hertz.

Table IV: Chemical Shift Anisotropy, Intrinsic Viscosity, and Lateral Diffusion Coefficient Values for PC and PE of Lipophorin Subspecies[a]

	particle radius[b] (nm)	$\Delta\sigma$ (ppm)	η' (P)	D_T (cm^2/s)
HDLp-W2				
PC	6.0	111.0 ± 5.0	0.569	4.61×10^{-8}
PE	6.0	106.0 ± 5.0	0.565	4.63×10^{-8}
HDLp-W1				
PC	7.0	91.0 ± 4.0	0.554	4.70×10^{-8}
PE	7.0	86.0 ± 4.0	0.550	4.73×10^{-8}
HDLp-A				
PC	8.0	74.0 ± 3.0	0.560	4.67×10^{-8}
PE	8.0	72.0 ± 3.0	0.566	4.63×10^{-8}
LDLp				
PC	12.0	43.0 ± 3.0	2.469	1.41×10^{-8}
PE	12.0	42.0 ± 3.0	2.248	1.52×10^{-8}

[a] Data were calculated as described in the text under Theory by curve fitting of the experimental data shown in Figure 2. [b] Assuming spherical particles.

η', diffusion coefficient, D_T, and chemical shift anisotropy, $\Delta\sigma$.

τ_c-Independent Line Width of Phospholipids in Lipophorins. The ^{31}P-NMR τ_c-independent portion of PC and PE line widths (C) for each lipophorin subspecies was obtained from plots of $1/T_2$ vs viscosity/temperature. For each subspecies the temperature range used was 0–39 °C, which results in a corresponding solvent viscosity range of 0.5–2.5 cP. For all lipophorins studied there was a linear relationship between $1/T_2$ and viscosity/temperature with correlation coefficients of 0.99 or greater. The consistentcy between our data and that of Cullis (1976) for egg PC in sonicated vesicles (15 Hz) indicates that C values, obtained by linear curve fitting of our data, are reasonable. However, on the basis of the differences in C among lipophorin subspecies and PC bilayer vesicles, it is clear that C is not a constant and needs to be determined experimentally for each system under study. As discussed under Theory, C is affected by instrumental parameters and the chemical shift anisotropy of the phosphate nucleus. While both these contributions are important determinants of C, it should be noted that the instrumental contribution to C for the same phospholipid in different lipophorins, or different phospholipids in same lipophorin, can be eliminated by using identical experimental conditions and processing parameters.

Phospholipid Chemical Shift Differences, η', $\Delta\sigma$, and D_T. The observation of two distinct resonances in *M. sexta* lipophorin is different from the results reported by Katagiri (1985) for *L. migratoria* lipophorin, which contains only a single resonance, assigned to PC. From Table I, it is clear that, in addition to PC, *M. sexta* lipophorins contain a considerable amount of PE. Rather than the possibility that a fraction of the phospholipid moiety exists in a unique chemical environment, the appearance of a second resonance in *M. sexta* lipophorins can be ascribed to formation, in PE, of an intramolecular seven-membered hydrogen-bonded ring structure involving the phosphate oxygen and its primary amine proton. By contrast, PC, which lacks the requisite dissociable proton in its head group, cannot form such a hydrogen-bonded structure. The deshielding effect of this hydrogen bond on the phosphate nucleus of PE causes its ^{31}P-NMR signal to shift downfield by 0.6 ppm compared to that of PC (Henderson et al., 1974). When different subspecies of lipophorin were compared, two resonances, separated by 0.6 ppm, were invariably present. The ratio of intensity of these resonances was reflected in the mass ratio of these two phospholipids, providing further support for this interpretation. The line widths of the PC and PE resonances in the same lipophorin species were comparable, but differences existed in the line widths of PC and PE in different subspecies. While HDLp-W2, HDLp-W1, and HDLp-A were similar, PC and PE line widths were broadened in LDLp, suggesting a more restrictive phospholipid environment in this species.

Values determined in the present study for the chemical shift anisotropy, intrinsic viscosity, and lateral diffusion coefficient for PC and PE in lipophorin subspecies are in good agreement with data reported by researchers for phospholipids in the other systems (Marsh, 1988; Edidin, 1974; Seelig, 1978; Seelig et al., 1981; Ghosh, 1988; Scherer & Seelig, 1989) including human plasma lipoproteins (Fenske et al., 1990). For example, D_T ranges from 2.3×10^{-8} cm^2/s for high-density lipoprotein (HDL) to 1.4×10^{-9} cm^2/s for low-density lipoprotein (LDL), whereas lipophorin D_T values obtained in the present study ranged from 4.6×10^{-8} cm^2/s for HDLp-W2 to 1.4×10^{-8} cm^2/s for LDLp. The observation that PC and PE moieties within the same lipophorin subspecies have similar intrinsic viscosity and chemical shift anisotropy values suggests these phospholipids exist in a homogeneous environment on the particle surface. Furthermore, the increased phospholipid η' in LDLp, compared to other subspecies, supports the concept that apoLp-III and/or DAG can induce an alteration in the phospholipid microenvironment.

Chemical shift anisotropy, $\Delta\sigma$, provides information about the orientational order of phospholipid head groups in the surface monolayer of lipophorin subspecies. Table IV indicates that, within the same subspecies, PC and PE $\Delta\sigma$ values are similar. Compared to other lipoproteins, LDLp has $\Delta\sigma$ values similiar to those observed for phospholipids in human LDL and very low density lipoprotein (Fenske et al., 1990). In a like manner the $\Delta\sigma$ of HDLp-A-associated phospholipids is similar to that of human HDL_2, while HDLp-W2 is similiar to human HDL_3. Among the different lipophorin subspecies, $\Delta\sigma$ values increase as particle protein weight percent increases. Furthermore, as the overall neutral lipid content of particles decreases, $\Delta\sigma$ increases. Thus it is likely that the observed differences in phospholipid $\Delta\sigma$ represent a combined effect of phospholipid interactions with protein, other surface lipids, and/or core lipid components. This effect, though, may be partially compensated by differences in lipophorin particle size which will affect particle tumbling and phospholipid packing within the surface monolayer.

Lipophorin Structure. Table V gives the content of DAG, PE, PC, and apolipophorins I, II, and III in different lipophorin subspecies which occur naturally during different life stages of this insect. Examination of these compositions reveals a consistent trend toward increasing DAG content from HDLp-W2 to HDLp-W1 to HDLp-A to LDLp. In fact, the weight percent of DAG in HDLp-A is approximately double that in

Table V: Composition and Properties of *M. sexta* Lipophorin Subspecies

	HDLp-W2[a]	HDLp-W1[a]	HDLp-A[b]	LDLp[b]
mol wt	550000	650000	786000	1500000
density (g/mL)	1.177	1.128	1.07	1.03
apoLp-I (240 kDa)	1	1	1	1
apoLp-II (85 kDa)	1	1	1	1
apoLp-III (18 kDa)	0	0	2	16
protein (%)	65.2	53.1	48.5	37.6
phospholipids (%)	18.9	23.3	14.0	7.1
DAG (%)	12.5	20.2	25.0	46.9
other lipids (%)	3.4	3.4	12.5	8.4
PL (mol/mol of Lp)	134	195	142	137
DAG (mol/mol of Lp)	100	193	289	1035
DAG/PL	0.75	1.0	2.04	7.66
apoLp-III/PL	0	0	0.014	0.117

[a] From Prasad et al. (1986). Molecular weights were estimated from the particle protein and lipid compositions. [b] From Ryan et al. (1986).

HDLp-W2 while that in LDLp is nearly 4 times that in HDLp-W2. Interestingly, these dramatic increases in particle DAG content are not accompanied by addition of significant quantities of other lipids. LDLp and its direct precursor, HDLp-A, however, do contain an additional apoprotein component, apoLp-III, not found on HDLp-W2 or HDLp-W1 (Ryan, 1990). Differences in $\Delta\nu_{1/2}$, η', and D_T of the PC and PE components of HDLp-W2, HDLp-W1, HDLp-A, and LDLp noted above (Tables II and IV) could conceivably arise from increased interaction of PC and PE with DAG as its concentration in various subspecies increases. Alternatively, phospholipid head-group interaction with apoLp-III may also be important in the observed differences. Either explanation, however, is compatible with the concept that PC and PE in the surface monolayer of LDLp are more restricted in their motion versus their motion in other subspecies.

It is generally accepted that DAG occurs naturally within membranes (Nishizuka, 1984). Studies with model bilayer membranes have shown, however, that depending on the type of phospholipid and the amount present, DAG can cause a lamellar to hexagonal II phase transition (Epand, 1985; Das & Rand, 1986). By analogy, it is plausible that DAG exists in the phospholipid monolayer of lipophorins. Unlike bilayer vesicles, lipophorin-associated DAG can partition into the hydrophobic core of the particle where it is largely segregated from the head groups of phospholipids in the surface monolayer. Indeed, it is likely that there is a dynamic equilibrium between DAG molecules in the particle core and the monolayer surface which may be influenced by the relative abundance of other surface components (Kawooya et al., 1991). On the basis of compositional analysis of various lipophorin subspecies, HDLp-W2 and HDLp-W1 have an apparent excess of these surface components. Thus it may be that the prevailing surface pressure on these particles forces DAG into the core. An important question raised by such an explanation pertains to HDLp-A, which contains two apoLp-III. It may be expected that interaction between phospholipid and apoLp-III would result in the appearance of additional or broadened resonances. Experimentally, however, only one set of relatively narrow resonances was found in the spectra of HDLp-A, suggesting that phospholipids might not interact with these two apoLp-III in HDLp-A. Indeed, it has been previously shown that these two apoLp-III are not exchangeable with the other fourteen which bind upon conversion to LDLp (Wells et al., 1987) nor can they be displaced by human apoA-I (Liu et al., 1991). Thus it is reasonable to suggest that apoLp-III in HDLp-A may interact more directly with the particle core and function more like integral apolipoprotein components than exchangeable, water-soluble, surface apolipoproteins.

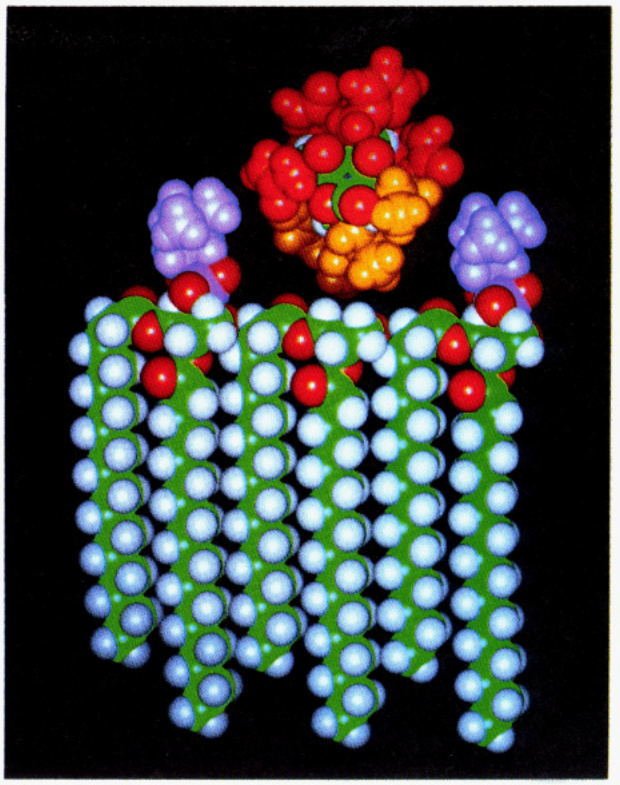

FIGURE 3: Space-filling molecular model depicting an interaction between an α-helical segment of apoLp-III (residues 36–45 of *L. migratoria* apoLp-III which is known to be in an amphipathic α-helical conformation from X-ray structure analysis; Breiter et al., 1991) and the surface monolayer of LDLp. Two phospholipid molecules (with the choline head group shown in lavender), intercalated by a single diacylglycerol, are shown. The hydrophobic face of the amphipathic helix segment (in yellow) is postulated to fill the gap created by the presence of diacylglycerol. The hydrophilic face of the helix (in red) is oriented toward the aqueous environment.

In LDLp, however, the DAG content increases by nearly 2-fold from that in HDLp-A (25%–46%), and an additional 14 molecules of apoLp-III associate with the particle (Ryan, 1990). Accompanying these compositional changes is an increase in particle diameter from 16 to ~24 nm. The observed increase in LDLp size in the absence of an increase in phospholipid content dictates that expansion of the surface creates a new lipid–water interface. This can be compensated for by a partitioning of DAG to the surface monolayer. Owing to its very small head group, however, increases in surface content of DAG will cause destabilization of the monolayer. We postulate that gaps created between neighboring phospholipids by intercalation of DAG can be stabilized by apoLp-III binding. Indeed the elongated amphipathic α-helical segments of apoLp-III (Brieter et al., 1991) appear well suited to perform this function (Kawooya et al., 1986; Singh et al., 1992). Subsequent interaction between apoLp-III and phospholipids could therefore explain the differences in $\Delta\nu_{1/2}$ η', and D_T in LDLp compared to other subspecies. A model illustrating a conceivable interaction of phospholipid, DAG, and apoLp-III on the surface of LDLp is shown in Figure 3. The model depicts an amphipathic α-helical segment of apoLp-III and two PC molecules intercalated by a single DAG. Molecular modeling studies revealed the hydrophobic face of the amphipathic α-helix of apoLp-III can fit comfortably between phospholipid head groups in the space created by the presence of DAG. In such a binding scenario, both the hydrophilic face of the α-helix and the phospholipid head

groups remain exposed to aqueous medium, thereby stabilizing the LDLp particle structure. This model could also apply to apoLp-III binding to DAG-enriched human LDL (Singh et al., 1992) and, possibly, mammalian water-soluble apolipoprotein binding to lipoprotein surfaces. It is clear, however, that further studies, probing the interactions of DAG, apoL-III, and phospholipid in lipophorin particles or in model membrane systems, will be required to reject or confirm this hypothesis.

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