

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/15063614>

Hydration and Localization of Diacylglycerol in the Insect Lipoprotein Lipophorin. A ^{13}C -NMR Study

ARTICLE *in* BIOCHEMISTRY · MARCH 1994

Impact Factor: 3.02 · DOI: 10.1021/bi00177a015 · Source: PubMed

CITATIONS

18

READS

9

4 AUTHORS, INCLUDING:



Mario Rivera

University of Kansas

82 PUBLICATIONS 1,900 CITATIONS

SEE PROFILE



F(rances) Ann Walker

The University of Arizona

242 PUBLICATIONS 8,714 CITATIONS

SEE PROFILE

Hydration and Localization of Diacylglycerol in the Insect Lipoprotein Lipophorin. A ^{13}C -NMR Study[†]

Jose L. Soulages,^{*,‡} Mario Rivera,[§] F. Ann Walker,[§] and Michael A. Wells[‡]

Departments of Biochemistry and Chemistry and Center for Insect Science, University of Arizona, Tucson, Arizona 85721

Received October 5, 1993; Revised Manuscript Received January 7, 1994[®]

ABSTRACT: In order to probe the organization of diacylglycerol (DG) in lipophorin, ^{13}C -enriched lipophorin was prepared for NMR investigations. We obtained ^{13}C -enriched lipophorin labeled exclusively in DG by feeding insects tobacco leaves coated with $[1-^{13}\text{C}]$ palmitic acid or $[1-^{13}\text{C}]$ oleic acid. Lipophorins enriched up to 5% with a $[^{13}\text{C}]$ fatty acid were obtained by this procedure. NMR studies of the isolated lipophorin DG showed that palmitic acid accumulates almost entirely (>90%) in the *sn*-1 position. Oleic acid was found equally distributed between the *sn*-1 and *sn*-2 positions, yielding a DG enriched equally at both positions. The ^{13}C -NMR spectra of both $[^{13}\text{C}]$ palmitate- and $[^{13}\text{C}]$ oleate-enriched lipophorins showed that DG had one narrow carbonyl resonance indicative of rapid motion. A comparative analysis of the ^{13}C carbonyl chemical shift data for DG in organic solvents, aqueous solutions, and dispersions with the DG carbonyl chemical shift of native lipophorin enriched in $[^{13}\text{C}]$ palmitate or $[^{13}\text{C}]$ oleate shows a high degree of water exclusion from the DG carbonyls in lipophorin. This result is consistent with the existence of a lipophorin lipid core containing most of the lipophorin DG. This study represents the first attempt to elucidate the organization of DG in lipophorin. The possibility of obtaining $[^{13}\text{C}]$ DG-enriched lipophorins, selectively enriched in one or both acyl chains of DG, should provide a powerful tool for further analysis of the organization and the dynamic properties of DG in native lipoproteins.

In insects, the process of dietary fat absorption requires the hydrolysis of triacylglycerols, which occurs in the lumen of the midgut (Hoffman & Downer, 1979; Weintraub & Tietz, 1973, 1978; Tsuchida & Wells, 1988). The FFA are absorbed and incorporated by the midgut cells into triacylglycerols, diacylglycerols (DG),¹ and phospholipids (PL). Most of the fatty acids are released from the midgut as DG, which are transported through the hemolymph by the major insect lipoprotein lipophorin (Weintraub & Tietz, 1978; Tsuchida & Wells, 1988; Bauerfeind & Komnick, 1992).

Whereas vertebrates rely on a battery of lipoproteins to effect lipid transport, insects use primarily a single type of lipoprotein, lipophorin, for lipid transport. Lipophorin transports PL, DG, hydrocarbons (HC), sterols, and free fatty acids among the insect tissues (Beenackers et al., 1985; Shapiro et al., 1988; Law & Wells, 1989; Van der Horst, 1990; Soulages & Wells, 1994a). The hallmarks of the lipophorin lipid composition are given by its high contents of 1,2-DG and long-chain (C_{20-40}) aliphatic hydrocarbons and its low contents of TG and cholesterol esters. All lipophorins contain two molecules of apolipoproteins per particle, one of apoLp-I, MW \approx 250 kDa, and one of apoLp-II, MW \approx 80 kDa. One interesting aspect of the structure of lipophorin is given by the ability of the particle to accommodate changes in the lipid content (20–50%) and composition (DG/PL molar ratios from \approx 0 to 2) without changes in the apoLp composition. In some stages of development and under certain physiological conditions, lipophorin is loaded with increasing amounts of DG,

reaching DG/PL molar ratios from 2 to \approx 10, and a third, exchangeable, apolipoprotein, apoLp-III (MW 18–20 kDa), binds to the lipoprotein particle (Beenackers et al., 1985; Wells et al., 1987). It has been determined that PLs reside on the surface of the lipoprotein (Katagiri, 1985) and HCs seem to be located in the interior of the lipophorin particle unexposed to water (Katagiri et al., 1985). On the basis of the chemical composition and density of lipophorins with moderate contents of DG and lacking apoLp-III, a lipophorin model was developed which suggested that there is a low content of DG on the lipoprotein surface (Soulages & Brenner, 1991; Soulages & Wells, 1994a). However, up to now, there were no experimental studies regarding the localization of DG in lipophorin.

The structure of lipophorin has been the subject of many studies and has been recently reviewed (Shapiro et al., 1988; Ryan, 1990; Soulages & Wells, 1994a). Due to the active metabolism of DG in insects and the high content of this lipid in lipophorin, it is important to know how this lipid is organized in the lipoprotein particle. In addition, the high content of DG found in lipophorins makes them an important model system in which to study the organization and the properties of DG in lipoprotein particles.

^{13}C NMR is a powerful tool for studying the organization of lipids in membranes and lipoproteins (Hamilton & Morriset, 1986). $[^{13}\text{C}]$ Cholesterol-enriched lipoproteins have been employed to determine the partition of cholesterol between the surface and the lipid core of the human LDL and HDL (Lund-Katz & Phillips, 1984, 1986). The high sensitivity of the ^{13}C carbonyl chemical shift to the ability of the solvent to form a H-bond was recognized early (Maciel & Natterstad, 1965; De Jeu, 1970) and exploited to study the structure and the organization of carbonyl-containing molecules in membranes (Schmidt et al., 1977; Hamilton & Small, 1981; Hamilton et al., 1982, 1991) and lipoproteins (Assman et al.,

[†] This work was supported by NIH Grant GM50008.

[‡] Department of Biochemistry and Center for Insect Science.

[§] Department of Chemistry.

[®] Abstract published in *Advance ACS Abstracts*, February 15, 1994.

¹ Abbreviations: ACN, acetonitrile; DMSO, dimethyl sulfoxide; PL, phospholipid; DG diacylglycerol; HC, hydrocarbon; diC₈-DG, dioctanoin; δ , chemical shift; T_1 , spin-lattice relaxation time; apoLp-I, apolipoprotein I; TLC, thin-layer chromatography.

1974). The main disadvantage of ^{13}C NMR resides in the low abundance of the isotope in natural materials, and for that reason, methods must be developed to obtain ^{13}C -enriched preparations.

The present study was conducted on a lipophorin species which does not contain apoLp-III, which has a DG/PL molar ratio of about 1, and in which DG represents about 16% of the lipoprotein weight (Prasad et al., 1986). This study shows that it is possible to obtain lipoproteins enriched exclusively in ^{13}C DG, thus providing an important tool for studying the largely unknown physical and chemical properties of DG in the lipophorin particle by NMR. In addition to the method for the preparation of ^{13}C -enriched lipoproteins, the present report shows substantial evidence for the location of DG in the larval lipophorin as well as for the selectivity of the acylation that takes place *in vivo* in the midgut of *Manduca sexta* larvae during the resynthesis of glycerides.

MATERIALS AND METHODS

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 containing 100 mM potassium phosphate buffer, pH 6.5, 2 mM EDTA, 1 mM diisopropyl phosphorofluoridate, and 20 mM glutathione. The hemolymph from adult insects was obtained by decapitation of insects after the injection of 1 mL of bleeding solution. In some cases, adult insects were bled 90 min after the injection of 100 pmol of adipokinetic hormone (AKH).

Preparation of *in Vivo* ^{13}C DG-Enriched Lipophorin. High-density lipophorin from larvae of *M. sexta* were enriched in ^{13}C DG by feeding 3-day-old fifth instar larvae with tobacco leaves coated with a thin film of 99% ^{13}C palmitic acid (Aldrich, Milwaukee, WI) or 99% ^{13}C oleic acid (Cambridge Isotopes Lab, Woburn, MA). The film of ^{13}C -palmitic acid contained 600 cpm of ^{14}C palmitic acid (Amersham Corp., Arlington Heights, IL) per microgram of fatty acid and was prepared by spreading an ethanolic solution of palmitic acid on the top of a small piece of the leaf. The ^{14}C palmitic acid was included to determine the percentage of labeled fatty acid recovered in lipophorin. The same procedure was employed with ^{13}C oleic acid containing ^3H -oleic acid (Amersham Corp., IL) or the following fatty acid mixtures: ^{13}C oleic acid/ ^{12}C linolenic acid (1:1 w/w) and ^{13}C oleic acid/ ^{12}C stearic acid/ ^{12}C myristic acid (1:0.5:0.5). After the solvent evaporated, each insect was fed approximately 250 mm² of leaf containing 400 μg of fatty acid. Sixty minutes after the insects ingested the piece of leaf, the hemolymph was obtained via a small incision in the second proleg and collected in a bleeding solution containing 50 mM potassium phosphate buffer, pH 6.5, 2 mM EDTA, 1 mM PMSF, 20 mM glutathione, and 0.02% NaN_3 . Lipophorin from about 100 insects was isolated by ultracentrifugation in a KBr gradient at 50 000 rpm, 5 $^\circ\text{C}$, for 16 h using a VTi 50 rotor (Shapiro et al., 1985) and the lipophorin band, approximate density 1.14 g/cm³, collected. The amount of ^{13}C palmitic acid which was incorporated into the lipophorin DG pool and the degree of enrichment were determined from the amount of radioactive palmitate found in lipophorin DG and the protein content of the lipophorin fraction, assuming that DG represents 15.7% of the lipophorin weight (Prasad et al., 1986), or from the radioactivity, the concentration of DG in the hemolymph (0.8 mg/mL), and the volume of hemolymph collected. The distribution of

radioactivity among the lipids of the lipophorin was determined after the separation of the lipids by TLC. Lipids were extracted from a lipophorin aliquot with a 2:1 (v/v) solution of chloroform-methanol (Folch et al., 1957) and analyzed by TLC on silica gel precoated plates (J. T. Baker) employing hexane-diethyl ether-acetic acid (70:30:2) as developing solvent mixture. Iodine-stained bands were marked and, after evaporation of the iodine, scrapped from the plate and counted in a liquid scintillation counter. Lipid classes were identified with reference to lipid standards separated on the same TLC plates.

Concentration and Fatty Acid Composition of the Hemolymph DG. Undiluted hemolymph was obtained from fifth instar larvae by puncture of the second proleg and collected in an Eppendorf tube containing 10 μL of a 20 mM glutathione solution. Total hemolymph volume was measured with a Hamilton microsyringe, and the samples were immediately transferred to glass conical tubes containing ditridecanoin, which was added as an internal standard for the quantitation of DG. Hemolymph lipids were extracted and separated by TLC, and the fatty acid composition was determined by GLC as described previously (Soulages & Wells, 1994b).

NMR Studies. The Fourier transform ^{13}C -NMR spectra of lipophorin and lipophorin lipids were obtained at 125.76 MHz in a Bruker AM 500 spectrometer or at 62.9 MHz in a Bruker AM 250 spectrometer. Both instruments operate in the quadrature detection mode. Measurements were performed at 25 ± 2 $^\circ\text{C}$ in 10-mm sample tubes, utilizing 10-mm broad-band probes. Spectra were acquired over 32K data points and a spectral width of 29.4 and 15.2 kHz at 125.76 and 62.9 MHz, respectively. The spectra were acquired with inverse gated decoupling. A relaxation delay of 2 s was introduced with the decoupler switched off. The decoupler was switched on during the acquisition time, 0.56 s, thus giving a recycling time of 2.56 s. The purpose of the decoupling scheme was to eliminate the NOE that normally arises upon broad-band decoupling.

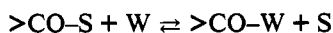
sn-1,2- and 1,3- ^{13}C dioctanoin (diC₈-DG) (Avanti Polar Lipids, Alabaster, AL) dissolved in CDCl_3 and CD_3OD were employed as references to make the signal assignment of the DG carbonyls. Deuterated solvents were obtained from Cambridge Isotopes Labs, Woburn, MA.

Lipophorin obtained from 100 insects was concentrated to 3 mL and the buffer replaced by 100 mM potassium phosphate, pH 6.5, containing 0.15 M NaCl and 50% D_2O in a Centriflo CF50 (Amicon, Danvers, MA). Lipophorin lipids were extracted with chloroform-methanol (2:1), dried under N_2 , and redissolved in CDCl_3 just before acquisition of the spectrum. In order to obtain the carbonyl chemical shift of dry DG, neutral lipids, mostly diacylglycerol and hydrocarbons, were obtained from the lipophorin of adult insects by silicic acid (200–400 mesh, Bio-Rad, Richmond, CA) chromatography. The total lipid extract was dissolved in chloroform and loaded onto a column of silicic acid equilibrated with chloroform, and neutral lipids were eluted with chloroform.

Chemical shifts of aqueous samples were referred indirectly to TMS, assuming an invariant chemical shift of 14.0 ppm for the methyl carbon (Hamilton et al., 1991). Chemical shifts of the diC₈-DG carbonyls in solutions of water-acetonitrile or water-dimethyl sulfoxide were also referred to the resonance of the methyl carbon.

In order to estimate the 100% hydration chemical shift value for monomeric DG, the following rationale was used. The apparent equilibrium and equilibrium constant for

carbonyl solvation are defined as:



where S = organic solvent and W = water.

$$K^{w/s} = (X_s/X_w)(Y_w/Y_s) \quad (1)$$

where X_s and X_w are the bulk molar fractions of organic solvent and water, respectively, and Y_w and Y_s are the molar fractions of the carbonyl–water and carbonyl–solvent pairs. Assuming that there are no contributions to the chemical shift of the carbonyl other than those arising from the carbonyl–water or carbonyl–solvent interaction, the observed chemical shift (δ_o) in a given mixture of water and organic solvent would be given by:

$$\delta_o = \delta_s Y_s + \delta_w Y_w \quad (2)$$

where δ_s and δ_w are the chemical shifts of the carbonyl in 100% organic solvent and 100% water, respectively. By rearrangement of eqs 1 and 2, the following linear relationship is obtained.

$$(\delta_o - \delta_s)^{-1} = (\delta_w - \delta_s)^{-1} + (\delta_w - \delta_s)^{-1} K^{-1} (X_s/X_w) \quad (3)$$

RESULTS

¹³C Enrichment of Lipophorin DG. One hour after the ingestion of 50 μg of [¹³C]fatty acid, offered as a thin lipid film on small pieces of tobacco leaves, an average ¹³C enrichment of lipophorin DG ranging between 3% and 5% was obtained. The lipids extracted from the lipophorin were analyzed by TLC, and essentially 100% of the radioactivity was found in the spot correspondent to 1,2-DG.

Figure 1 shows the complete proton-decoupled ¹³C spectrum obtained with the lipids extracted from the lipophorin of control insects and the complete spectra obtained with lipophorin lipids enriched with [¹³C]palmitic or [¹³C]oleic acid. Although, under our experimental conditions (recycling time < 5 T_1) and due to the different relaxation times of the ¹³C in different chemical groups, the NMR intensities are not a measure of the abundance of the different chemical groups, they are still useful for comparing the degree of enrichment of a particular chemical group. The comparison of the relative intensities of the resonances of the carbonyl groups (173.8–173.4 ppm) with those corresponding to any other unenriched chemical group (CH_3 at 14 ppm, $-\text{CH}=\text{CH}-$ at 128 ppm, or the methylene envelope around 29 ppm) in Figure 1 (left panel) clearly confirmed the relatively high degree of ¹³C carbonyl enrichment achieved with either [¹³C]oleic acid or [¹³C]palmitic acid.

Table 1 shows the fatty acid composition of DG from the hemolymph of fifth instar larvae. Under our rearing conditions, palmitic acid represents 43.5% of the fatty acids and oleic acid accounts for about 15%. Despite this difference in fatty acid content, we did not observe a higher enrichment with palmitic acid than with oleic acid.

The signal assignments for the resonances of the *sn*-1 and *sn*-2 carbonyls of 1,2-DG and the chemical shifts of the magnetically equivalent carbonyls of 1,3-DG, in organic solvents, have been previously reported (Hamilton et al., 1991). Our results with 1,2-diC₈-DG and 1,3-diC₈-DG confirm those data, and as shown in Figure 1, right panel A and B, and Table 2, the resonances of the carbonyls at the *sn*-1 (173.8 ppm) and *sn*-2 (173.4 ppm) positions, as well as the chemical shifts of the 1,3-carbonyls of 1,3-DG (173.9 ppm), are very

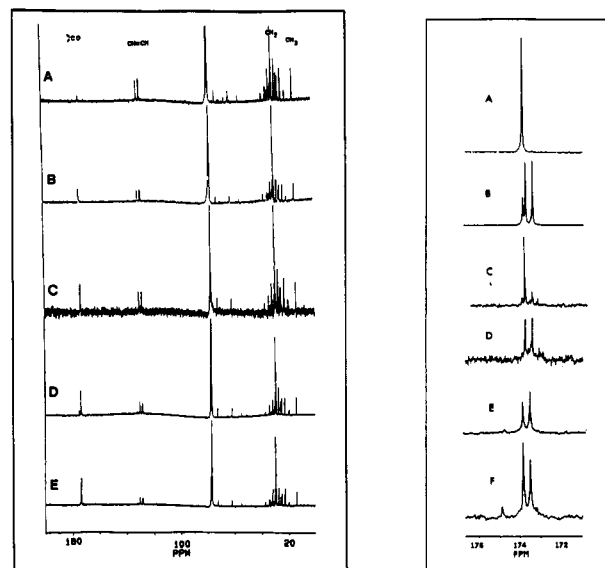


FIGURE 1: Left panel: H-decoupled ¹³C-NMR spectra of (A) unenriched lipophorin lipids, (B) [¹³C]palmitic-acid-enriched lipophorin lipids, (C) [¹³C]oleic-acid-enriched lipophorin lipids, (D) lipophorin lipids obtained after feeding insects with a 1:1 mixture of [¹³C]oleic acid and [¹²C]linolenic acid, and (E) lipophorin lipids obtained after feeding insects with a 1:1 mixture of [¹³C]oleic acid and [¹²C]saturated fatty acids, 14:0 + 18:0. The spectra were taken in CCl₃D at 25 °C, and the chemical shifts are referred to TMS. All the spectra were obtained with gated decoupling and a recycling time of 2.6 s so that the NOE was suppressed. Spectra were processed with an exponential multiplication and a line broadening of 2 Hz. The number of scans was variable and ranged from 1000 to 6000. Right panel: expanded carbonyl regions of the ¹³C-NMR spectra of (A) [1,1'-¹³C]-1,3-diC₈-DG, (B) [1,1'-¹³C]-1,2-diC₈-DG, (C) [¹³C]-palmitic-acid-enriched lipophorin lipids, (D) [¹³C]oleic-acid-enriched lipophorin lipids, (E) lipophorin lipids obtained after feeding insects with a 1:1 mixture of [¹³C]oleic acid and [¹²C]linolenic acid, and (F) lipophorin lipids obtained after feeding insects with a 1:1 mixture of [¹³C]oleic acid and [¹²C]saturated fatty acids, 14:0 + 18:0.

Table 1: Fatty Acid Composition of the Hemolymph DG of Fifth Instar Larvae^a

fatty acids					
14:0	16:0	18:0	18:1	18:2	other
1.1 ± 1.0	43.5 ± 5.0	9.3 ± 2.6	14.7 ± 2.0	7.2 ± 3.7	24.3 ± 4.7

^a Data are the average of four determinations ± SD. "Other" fatty acids contain the sum of long-chain and polyunsaturated fatty acids which were not identified.

well resolved in chloroform. Figure 1, right panel, also shows the expanded carbonyl region of the spectra shown in the left panel of Figure 1. It can be seen that [¹³C]oleic acid was almost equally distributed between positions 1 and 2 of the glycerol backbone of DG, right panel D, whereas more than 90% of the [¹³C]palmitic acid was found at position 1 of the DG, Figure 1, right panel C. The fact that palmitic acid is almost exclusively esterified to the 1 position of the 1,2-DG indicates that insects are employing endogenous fatty acids to acylate position 2 of glycerol. The source of endogenous fatty acids has not been studied. However, because the insects employed had been eating their regular diet until minutes before the experiment, the fatty acids may come from the diet present in the midgut lumen at the time of the [¹³C]fatty acid ingestion or from an intracellular pool of fatty acids stored in the midgut epithelium. In order to study further the specificity of the acylation that takes place in the midgut, we fed insects with tobacco leaves covered with a 50% (w/w) mixture of [¹³C]oleic acid and [¹²C]linolenic acid or with a mixture of [¹³C]oleic acid and [¹²C]saturated fatty acids, myristic plus

Table 2: Chemical Shifts of DG Carbonyls in Different Systems^a

	1,2-diC ₈ -DG		lipophorin DG		1,2-diC ₁₄ -DG ^d	
	sn-1	sn-2	sn-1	sn-2	sn-1	sn-2
CCl ₄	172.48	172.09			172.03	171.62
DMSO- <i>d</i> ₆	172.55	172.36				
CDCl ₃	173.78	173.42	173.81	173.47	173.80	173.43
ACN- <i>d</i> ₃	174.15	173.94				
CD ₃ OD	174.98	174.82				
neat					172.95	172.85
dry DG-HC			172.82	172.70		
aqueous SDS	174.41	174.36			174.61	174.56
lipophorin			173.16 ^b	173.16 ^b		
			173.25 ^c	173.25 ^c		

^a The spectra of 1,2-diC₈-DG in organic solvents were obtained in solutions containing a 2.5:97.5 mixture of ¹³C-enriched:natural abundance diC₈-DG, at a total lipid concentration of 8–10 mg/mL. ^b Carbonyl chemical shift of [¹³C]oleic-acid-enriched lipophorin. ^c Carbonyl chemical shift of [¹³C]palmitic-acid-enriched lipophorin. ^d Data for diC₁₄-DG were extracted from Hamilton et al. (1991).

stearic acids. Right panel E of Figure 1 shows that, as a result of the presence of linolenic acid, the distribution of oleic acid is slightly shifted to position 1 of the glycerol backbone. Conversely, the mixture of oleic acid and saturated fatty acid promoted a slight accumulation of oleic acid at position 2 (panel F of Figure 1). Assuming that linolenic acid could acylate either position of the 1,2-DG, we expected no effect of linolenic acid on the distribution of oleic acid. The fact that a small, but significant, decrease in the acylation of position 2 by oleic acid was observed in the presence of linolenic acid indicates a preference of the acyltransferases to acylate position 2 with linolenic acid. A similar rationale can explain the results observed with the mixture of oleic acid and saturated fatty acids.

Figure 2 shows the complete ¹³C-NMR spectra of lipophorins enriched with [¹³C]palmitic acid (A) and [¹³C]oleic acid (B). The higher relative intensity of the carbonyl to the methyl carbons that is found when the spectra of lipophorin lipids is compared to the spectra of the lipids in chloroform, Figure 1, is likely due to the faster relaxation rate of the carbonyl in the lipoprotein. The carbonyl region of the spectrum shows only one resonance at 173.25 ppm for [¹³C]-palmitic-acid-enriched lipophorin. Only one carbonyl resonance and at a similar chemical shift, 173.16 ppm, is also observed with [¹³C]oleic-acid-enriched lipophorins. The resonances arising from positions 1 and 2 of [¹³C]oleic-acid-enriched DG were clearly resolved and of the same intensity when the lipids were extracted from the lipophorin and the spectrum was obtained in organic solvents. Therefore, the single resonance found in lipophorin is the result of the collapse of the two carbonyl resonances. The narrow line widths that are observed, at 25 °C, in both [¹³C]oleic-acid- and [¹³C]-palmitic-acid-enriched lipophorins, $u_{1/2}$ = 15 and 11 Hz, respectively, indicate a high mobility of the DG molecules in the lipoprotein and suggest that the chemical shifts of the two carbonyls in oleic-acid-enriched lipophorins resonate at very close frequencies.

Solvation Effects on the DG Carbonyl Chemical Shift. Solvation effects on the ¹³C chemical shift of the DG carbonyl resonance were investigated in organic solvents of different polarity and H-bonding capacity as well as in solutions of DG in water-ACN and water-DMSO mixtures and aqueous dispersions of DG in SDS micelles. Table 2 shows the results of this study as well as those previously reported by Hamilton et al. (1991) for diC₁₄-DG. The chemical shifts of the carbonyls of diC₈-DG showed similar values to those reported for diC₁₄-DG in carbon tetrachloride, chloroform, and SDS

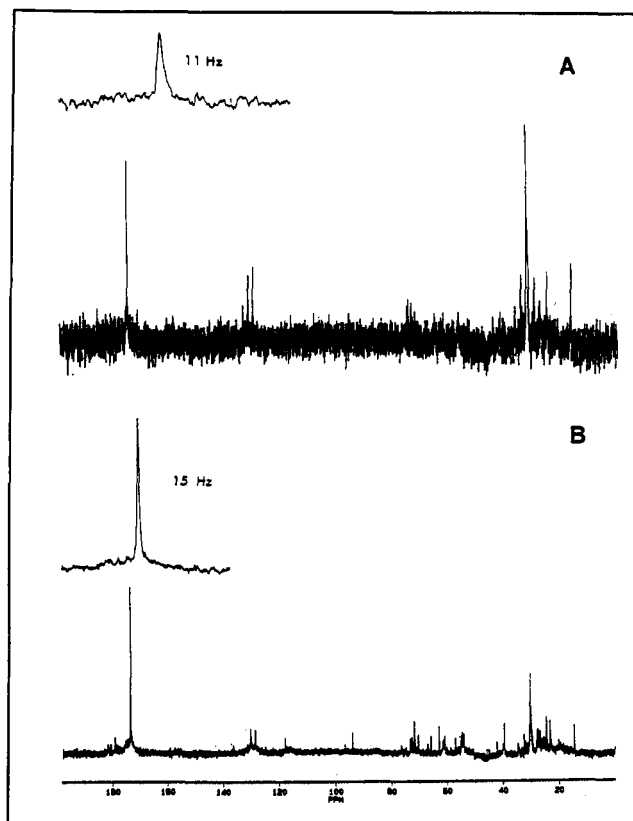


FIGURE 2: (A) ¹³C-NMR spectrum of native larval HDLp *in vivo* enriched with [¹³C]palmitic acid and (B) ¹³C-NMR spectrum of native lipophorin *in vivo* enriched with [¹³C]oleic acid; insets show the expanded carbonyl region of the respective complete spectra. The temperature of the samples was 25 ± 2 °C.

micelles. These data show that there is no correlation between the dielectric constant of the solvent and the carbonyl chemical shift of DG. A similar lack of correlation was also observed when the carbonyl chemical shift of acetone in different solvents was studied (De Jeu, 1970). However, there seems to be a correlation between the H-bonding ability of the solvent and the carbonyl chemical shift. The carbonyl chemical shift of DG in a dry mixture of DG and hydrocarbons (172.8 and 172.7 ppm), extracted from lipophorin, containing about 90% DG and 10% HC, was similar to the chemical shift reported for neat DG (172.95 and 172.85 ppm) (Hamilton et al., 1991).

Because long-chain DGs are highly insoluble in water, it is not possible to determine directly the chemical shift of the DG carbonyl in water in the absence of a macromolecular structure. It has been assumed that SDS-DG micelles represent a good model system to study maximum carbonyl hydration (Hamilton et al., 1991). However, the possibility that some degree of water exclusion could occur in the micelle can not be strictly ruled out. In order to obtain additional data on the chemical shift of highly hydrated DG carbonyls, we determined the chemical shift of diC₈-DG in mixtures of ACN-water and DMSO-water of increasing water content. Due to the higher solubility of the short-acyl-chain DG in water or other polar solvents, the use of diC₈-DG permits an analysis of the behavior of the chemical shift of the carbonyls in monomeric DG, covering a wide range of water content. These data provide an extrapolated chemical shift value which would represent maximum hydration of the carbonyl and excludes the possible effect of the micellar structure on the chemical shift of the carbonyl. Figure 3 shows a double reciprocal plot, according to eq 3, of the variation of the carbonyl chemical shift against the water content of the solvent.

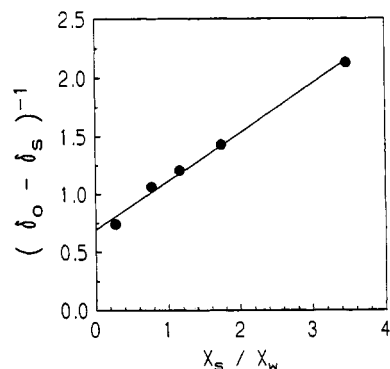


FIGURE 3: Double reciprocal plot of $\delta_0 - \delta_s$ vs X_s/X_w for ACN-water-diC₈-DG solutions. δ_0 and δ_s are the chemical shifts observed in water-solvent mixtures and in 100% organic solvent, respectively. X_s and X_w are the molar fractions of solvent and water, respectively. Chemical shifts of the *sn*-1 carbonyl were employed in the plot and referred to the resonance of the methyl carbon as indicated in Materials and Methods. A linear regression was employed to fit the data.

As expected, the carbonyl chemical shifts of 1,2-diC₈-DG in mixtures of ACN-water and DMSO-water (Figures 3 and 4) increases as the water content increases. In the ACN-water system, a reasonably good linear behavior allowed an extrapolation to 100% water (Figure 3). However, a nonlinear relationship was found in DMSO-water solutions. From the ACN-water system, an extrapolated value for 100% hydration of the carbonyl group gave a chemical shift of 175.2 ppm for the carbonyl at the *sn*-1 position in monomeric DG. This value is close to the carbonyl chemical shift values observed in SDS-DG micelles. However, there was a downfield difference between the 100% hydration chemical shift values obtained from the plot of Figure 3 and the chemical shift observed in SDS-DG micelles. This result suggests that in SDS-DG micelles some degree of water exclusion from the carbonyl occurs. If the chemical shift of the DG carbonyl in carbon tetrachloride is taken as a reference for zero hydration, then a degree of water exclusion of about 15% is calculated to occur in SDS-DG micelles.

The DMSO-water system allowed us to observe, simultaneously, the chemical shifts of the DG carbonyl in a micellar, aggregated state and the monomeric state. The high critical micellar concentration of diC₈-DG in DMSO or DMSO-water solutions gave us the chance to observe the carbonyl chemical shifts in micelles and in solutions having different water contents. Figure 4 shows the effect of increasing water content on the spectra of diC₈-DG. As shown in the first three spectra of Figure 4, for X_w of 0, 0.29, and 0.48 and a lipid concentration of 22 mM, diC₈-DG remains soluble up to a molar fraction of water of about 0.5. The solubility of diC₈-DG decreases with increasing water content, and the lipid adopts a micellar phase, which coexists with monomeric diC₈-DG. The high solubility of the lipid in DMSO-water allowed the simultaneous observation of the carbonyl resonances of DG in the soluble and micellar phase, giving spectra characterized by the presence of four carbonyl resonances (see spectrum at 22 mM diC₈-DG and 57% water). As expected, the concentration of water at which the formation of the micellar phase is observed is dependent on the concentration of the lipid. It can be seen, Figure 4, that decreasing the concentration of diC₈-DG from 22 to 11 mM promotes the disappearance of the micellar carbonyl resonances. Although we have not covered a very extensive range of diC₈-DG and water concentrations, from our data approximate critical micellar concentrations of diC₈-DG in DMSO-water of 22, 11, and 3.5 mM were observed at molar

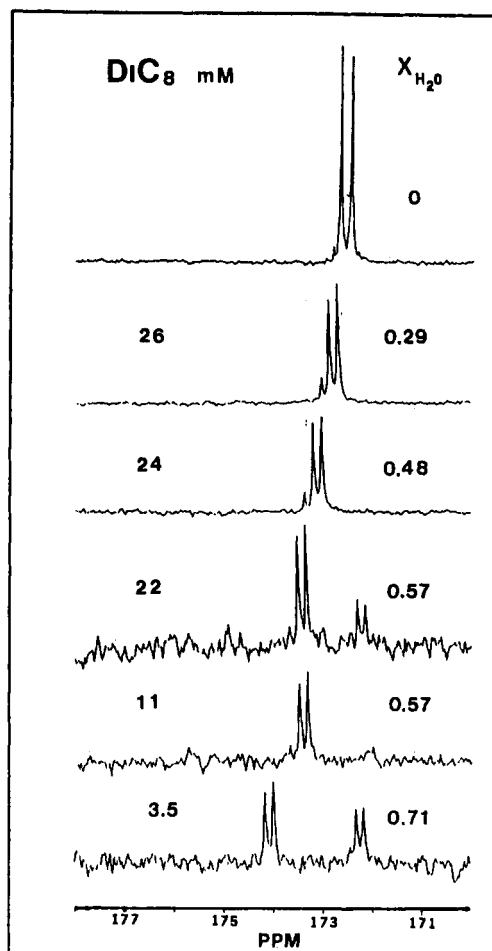


FIGURE 4: Carbonyl region of the spectra of 1,2-diC₈-DG in DMSO-water mixtures. The correspondent concentrations of water (molar fraction) and diC₈-DG (mM) are indicated in the right and left sides of the plots, respectively.

fractions of water of 0.57, 0.63 (data not shown), and 0.71, respectively. The coexistence of the monomeric and micellar diC₈-DG seems to be very stable, and 3 days after the sample preparation, the spectrum obtained was essentially identical to the spectrum obtained with a fresh sample. In the presence of micelles, the chemical shift of the monomer follows the tendency observed in the absence of lipid aggregates, i.e., its chemical shift increases as the water content increases. Conversely, the chemical shift of the diC₈-DG carbonyl in the micellar lipid was shifted to lower frequency and reached a value which was independent of the water content of the solvent mixture. The chemical shift in the aggregate is lower than that observed in 100% DMSO and close to that found in neat DG, suggesting that in the micelle DMSO was excluded from interacting with the carbonyl groups. Interestingly, when referred to the methyl carbon, the chemical shifts of the methylene envelope are almost independent of the water content of the solution, and thus, with the exception of the carbonyl, only single resonances are observed for the remaining chemical groups when two phases are present in the system. This observation corroborates the high sensitivity of the carbonyl chemical shift to the chemical nature of the solvents.

The chemical shift of lipophorin DG carbonyls (173.2 ppm) falls between the chemical shifts of the DG carbonyls observed in chloroform (173.8–173.4 ppm) and carbon tetrachloride (171.6–172.1 ppm), indicating a low degree of hydration. The chemical shifts of DG carbonyls in lipophorin are slightly downfield from the chemical shifts of the dry DG-HC mixture

(172.82–172.70 ppm), which might represent the lipid core of lipophorin, or neat DG (172.85–172.95 ppm), indicating a slightly higher H-bonded interaction of DG in lipophorin than that found in dry lipids. As for any other lipoprotein, lipophorin can be envisaged as having two pools of lipid, one at the surface of the particle, which would be hydrated, and one that would be interior and not exposed to water. The carbonyl chemical shift of a putative pool of surface DG could be compared to the chemical shift of DG in aqueous dispersions of SDS–DG micelles or to the value obtained from mixtures of ACN–water. On the other hand, the chemical shift of the carbonyl of DG in an internal lipoprotein lipid pool, which in the case of lipophorin would be composed of DG and small amounts of hydrocarbons, can be related to the chemical shift observed in neat DG or in the dry DG–HC mixture obtained from lipophorin. The presence of only one carbonyl resonance in native lipophorin would indicate either that there is only one DG pool in lipophorin or that, if more than one pool exists, in the NMR time scale, they are in fast exchange. If the aqueous SDS–DG and dry DG (or dry DG–HC) samples are taken as reference states for maximum and minimum hydration, then, from the chemical shift of the DG carbonyl found in native lipophorins, it can be calculated that only a small fraction of the DG molecules, less than 20%, would be as hydrated as the DG in SDS micelles and more than 80% of the DG molecules would be represented by a system similar to that of neat DG.

If, as suggested by the narrow line width of the carbonyl resonances observed in lipophorin, the existence of two DG pools in fast exchange is considered, one on the surface and the other internal, then it is concluded that less than 20% of the lipophorin DG would reside on the lipophorin surface.

DISCUSSION

When compared to ^1H -NMR spectroscopy, ^{13}C -NMR spectroscopy offers distinct advantages for the study of complexes systems like lipoproteins (Hamilton & Morriset, 1986). The study of the ^{13}C spin-lattice relaxation time provides a nonperturbing probe to analyze the internal mobility of organic molecules, and it has been used to study the dynamics of lipids in membranes and lipoproteins. The main disadvantage of ^{13}C NMR resides in the low abundance of the isotope in natural materials, and for that reason, methods must be developed to obtain ^{13}C -enriched preparations. In some cases, relaxation reagents have been useful in studying the topology of lipids in membranes and lipoproteins. Unfortunately, the use of these reagents to study the localization of DG did not work in our system because most of the reagents employed did not show any effect on the DG carbonyl resonance, at least until their concentration was high enough to precipitate the lipoprotein.

In this report, we have shown that it is possible to obtain lipophorin highly and selectively enriched in ^{13}C DG. In addition, we have shown that it is possible to enrich selectively with a ^{13}C fatty acyl chain at the *sn*-1 glycerol carbon by employing palmitic acid and at both acyl positions of DG by employing oleic acid. The results also indicate that it is likely that, by employing a polyunsaturated fatty acid, the acyl chain which esterifies the *sn*-2 carbon of glycerol could be selectively enriched.

The localization of DG in the lipophorin particle is a subject yet under discussion (Soulages & Wells, 1994a). A composition–structure correlation model developed for lipophorins that do not contain apoLp-III seems to be consistent with a majority of the DG molecules residing in the hydrophobic

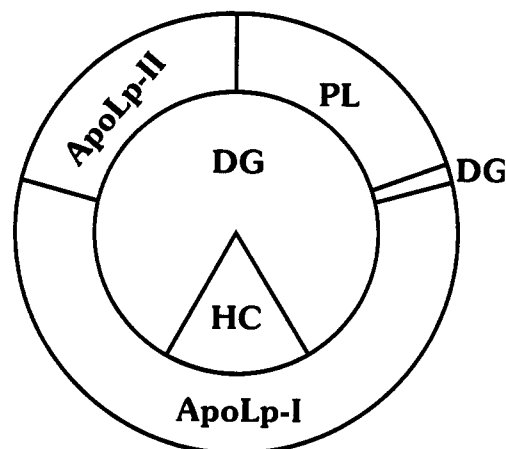


FIGURE 5: Schematic representation of a lipophorin particle. The space-filling scheme was based on the lipid composition data of Prasad et al. (1986), assuming the apolipoproteins and phospholipids form an outer shell of 20-Å thickness. The apparent area occupied by the apolipoproteins has been estimated to be 32 000 Å² (Soulages & Brenner, 1991), which accounts for about 80% of the surface area of the lipophorin of this study (radius = 56.2 Å). HC and DG would reside mostly in the lipoprotein core. The polar groups of the phospholipids are all exposed to the aqueous phase (Katagiri, 1985), and only a small fraction of the lipophorin DG would reside on the lipoprotein surface.

lipid core (Soulages & Brenner, 1991). The composition–structure correlation showed that the content of PLs is linearly related to the size of the lipoprotein and indicated that, at the surface of the lipophorin, apolipoproteins would occupy a constant area of about 32 000 Å². From the compositional data on the larval lipophorin (Prasad et al., 1986), the area and volume occupied by the apolipoproteins (Soulages & Brenner, 1991), and the virtual absence of DG on the lipoprotein surface that is inferred from the current study, a structural scheme of the lipophorin particle has been depicted in Figure 5.

The comparison of the chemical shift of DG carbonyls in lipophorin with the chemical shifts of the DG carbonyls in carbon tetrachloride, which serves as a reference for a medium of low polarity and non-H-bond ability, suggests that the lipophorin DG carbonyl groups are at least partially H-bonded. A shift to higher frequency of the DG carbonyl resonance from its position in carbon tetrachloride and the fusion of the carbonyl peaks of DG were previously observed for DG incorporated into liposomes of phosphatidylcholine and attributed to carbonyl hydration and the different degree of hydration of the *sn*-1 and *sn*-2 carbonyls of DG in liposomes (Hamilton et al., 1991). The chemical shift of the carbonyl resonance observed in both ^{13}C palmitic-acid- and ^{13}C oleic-acid-enriched lipophorins indicates a low average degree of hydration of the DG carbonyls in these lipoproteins. Because of the high content of DG in lipophorin and the condensed nature of the system, the existence of a DG-rich phase is very likely. Thus, the coalescence of the *sn*-1 and *sn*-2 resonances observed in ^{13}C oleic-acid-enriched lipophorin could be derived not from a difference in carbonyl hydration but rather from interactions of the carbonyls with hydrogens other than those from water. A possible explanation for the fusion of the resonances could be the formation of intramolecular H-bonds between the free hydroxyl group and the *sn*-2 carbonyl of the DG. In fact, the carbonyl chemical shifts observed in neat DG or dry DG–HC are very close to the chemical shift of DG carbonyls in lipophorin and downfield from the chemical shift of DG carbonyls in CCl₄, indicating that in those systems the carbonyls are partially H-bonded. The decrease in the

separation between the chemical shifts of the *sn*-1 and *sn*-2 carbonyls that is observed in dry DG preparations, when compared to either CCl₄ or CDCl₃ solutions of DG, might be a result of the higher chance of H-bonding between the free hydroxyl of the DG molecule and the *sn*-2 carbonyl. The intramolecular H-bond would favor the dehydration of the hydroxyl and, therefore, an internal localization of the DG molecule.

The perturbing effect of DG on the packing of lipids has been observed in natural and artificial membranes (Dawson et al., 1983; Das & Rand, 1986; Hamilton et al., 1991). In a recent study, we demonstrated that the incorporation of DG into larval lipophorin promotes a large lipid disorder and the binding of apoLp-III (Soulages & Wells, 1994c). In addition, the incorporation of DG in the absence of apoLp-III provokes the aggregation of the lipophorin, which further demonstrates the destabilizing effect of DG on the lipophorin surface and is also consistent with the low content of surface DG inferred from this study.

REFERENCES

- Assmann, G., Highet, R. J., Sokoloski, E. A., & Brewer, H. B., Jr. (1974) *Proc. Natl. Acad. Sci. U.S.A.* 71, 3701–3705.
- Bauerfeind, R., & Komnick, H. (1992) *J. Insect Physiol.* 38, 147–160.
- Beenackers, A. M. Th., Van der Horst, D. J., & Van Marrewijk, W. J. A. (1985) *Prog. Lipid Res.* 24, 19–67.
- Das, S., & Rand, R. P. (1986) *Biochemistry* 26, 2882–2889.
- Dawson, R. M. C., Hemington, N. L., & Irvine, R. F. (1983) *Biochem. Biophys. Res. Commun.* 117, 196–201.
- De Jeu, W. H. (1970) *J. Phys. Chem.* 74, 822–830.
- Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* 226, 497–509.
- Hamilton, J. A., & Small, D. M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 6878–6882.
- Hamilton, J. A., & Small, D. M. (1982) *J. Biol. Chem.* 257, 7318–7321.
- Hamilton, J. A., & Morrisett, J. D. (1986) *Methods Enzymol.* 128, 472–515.
- Hamilton, J. A., Bhamidipati, S. P., Kodali, D. R., & Small, D. M. (1991) *J. Biol. Chem.* 266, 1177–1186.
- Hoffman, A. G., & Downer, R. G. H. (1979) *Insect Biochem.* 9, 129–134.
- Hoffman, A. G., & Downer, R. G. H. (1979) *Can. J. Zool.* 54, 1165–1171.
- Katagiri, C. (1985) *Biochim. Biophys. Acta* 834, 139–143.
- Katagiri, C., Kimura, J., & Murase, N. (1985) *J. Biol. Chem.* 260, 13490–13495.
- Lund-Katz, S., & Phillips, M. C. (1984) *Biochemistry* 23, 1130–1138.
- Lund-Katz, S., & Phillips, M. C. (1986) *Biochemistry* 25, 1562–1568.
- Maciel, G. E., & Natterstad, J. J. (1965) *J. Chem. Phys.* 42, 2752–2759.
- Prasad, S. V., Ryan, R. O., Law, J. H., & Wells, M. A. (1986) *J. Biol. Chem.* 261, 558–562.
- Ryan, R. O. (1990) *J. Lipid Res.* 31, 1725–1739.
- Schmidt, C. F., Barenholz, Y., Huang, C., & Thompson, T. E. (1977) *Biochemistry* 16, 3948–3954.
- 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.
- 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) *Insect Biochem. Mol. Biol.* 24, 79–86.
- Soulages, J. L., & Wells, M. A. (1994c) *Biochemistry* (in press).
- Tsuchida, K., & Wells, M. A. (1988) *Insect Biochem.* 18, 263–268.
- Van der Horst, D. J. (1990) *Biochim. Biophys. Acta* 1047, 195–211.
- Weintraub, H., & Tietz, A. (1973) *Biochim. Biophys. Acta* 306, 31–41.
- Weintraub, H., & Tietz, A. (1978) *Insect Biochem.* 8, 267–274.