Lipoprotein-X: Carbon-13 Nuclear Magnetic Resonance Studies on Native, Reconstituted, and Model Systems†

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ABSTRACT: Lipoprotein-X (LP-X), a lipoprotein isolated from human cholestatic plasma by ethanol–acetate precipitation and zonal ultracentrifugation, has been studied by ^{13}C NMR at 67.9 MHz. Spectra of LP-X and its three subfractions are markedly different from those of normal human high-density lipoprotein$_3$ (HDL_3) or low-density lipoprotein (LDL). Spectra of LP-X are characterized by the presence of unusually broad resonance lines, especially those attributable to C6 of unesterified cholesterol (160–260 Hz) and to Cβ of phospholipid glyceride (240–290 Hz). In contrast, the CH_2O, CH_2N, and $N(CH_3)_3$ choline resonances have line widths comparable to those of normal LDL and HDL_3. For the subfraction LP-X_1, spin–lattice relaxation times (T_1) of the fatty acyl olefin resonances at 129.8 and 128.0 ppm and of the unesterified cholesterol C6 at 120.1 ppm were measured to be 675, 766, and 162 ms, respectively. These times are comparable to those measured for the corresponding resonances in single bilayer vesicles whose lipid composition approximates that of LP-X. The three LP-X subfractions isolated by zonal ultracentrifugation gave spectra which are identical, within experimental error, as judged qualitatively from their appearance and quantitatively from the line widths of selected resonances. In addition, ^{13}C NMR spectra of sonicated total LP-X lipids are similar to spectra of the intact native lipoprotein. This study suggests (a) that motions of lipids in LP-X as probed by ^{13}C NMR are similar to the motions of lipids found in model vesicular systems, (b) that the motions of the cholesterol rings and phospholipid fatty acyl chains are significantly more restricted in LP-X than in HDL_3 and LDL, and (c) that the motions of the phosphoryl moieties in all three systems are similar.

Lipoprotein-X[1] is an abnormal lipoprotein frequently found in the plasma of patients suffering from obstructive liver disease. This lipoprotein bands in the density range *d* 1.006–1.063, where normal LDL is also found. LP-X is distinguished from all other lipoproteins by its unusually high content of phospholipids (~65%) and cholesterol (~25%). It possesses low proportions of cholesteryl ester (~1%), triglycerides (~2%), and protein (~5%). The unique chemical composition of LP-X undoubtedly accounts for its physical and metabolic properties which are distinct from other lipoproteins. When visualized by negative-stain electron microscopy, these particles appear as disks with a major axis of 400–600 Å and a minor axis of ~100 Å. Preliminary X-ray scattering studies suggested that the LP-X particle is a flattened vesicle whose wall is a continuous lipid bilayer (Hamilton et al., 1971). Subsequently, a more detailed X-ray scattering study by Laggner et al. (1977) firmly established that these particles are spherical vesicular structures that contain an internal aqueous space. The stacking of LP-X particles observed by negative-stain electron microscopy has not been observed by X-ray scattering and is currently regarded as artifactual. The essential features of bilayer structure in LP-X have also been observed by Hauser et al. (1977) using electron microscopic and magnetic resonance techniques.

The high proportion of unesterified cholesterol in LP-X confers an abnormally low fluidity on these particles (Patsch et al., 1977a). A physiological consequence of this low level of lipid fluidity is the low efficacy with which lecithin:cholesterol acyltransferase acts on these particles (Patsch et al., 1977b). Although the studies cited above have provided information about the overall macroscopic structure of LP-X, they have been of limited value in understanding the microscopic molecular interactions occurring in these particles. The purpose of this present study is to provide this type of information and gain a clearer understanding of LP-X structure on a molecular level. In the first paper of this two-part series, we describe a study of LP-X and its principal lipid components by high-field natural abundance ^{13}C NMR.

Experimental Section

Materials. Lipoprotein-X was obtained from the plasma of several cholestatic patients by a combination of treatment with cold ethanolic acetate buffer and zonal ultracentrifugation (Patsch et al., 1977a). The subfractions LP-X_1, LP-X_2, and LP-X_3 were obtained in homogeneous form by recentrifugation. Crude LDL and HDL_3 were obtained from normal, fasting male donors by sequential centrifugation of the plasma

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[1] Abbreviations used: LP-X_t, total, unfractionated lipoprotein-X; LP-X_1, LP-X_2, and LP-X_3, subfractions of LP-X_t obtained by Cohn fractionation and zonal ultracentrifugation; LDL, low-density lipoproteins isolated at *d* 1.019–1.063; HDL, high-density lipoproteins isolated at *d* 1.063–1.210; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; T_1, spin–lattice relaxation time; Cα (Cβ), the glyceride CH_2 (CH) of phosphatidylcholine which bears a fatty acyl chain; Cγ, the glyceride CH_2 carbon which bears the phosphorylcholine moiety; Me_4Si, tetramethylsilane; ppm, parts per million; DPH, 1,6-diphenyl-1,3,5-hexatriene; EYPC, egg yolk phosphatidylcholine; *d*, density in g/mL; UC, unesterified cholesterol.

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at *d* 1.019–1.063 and *d* 1.125–1.210, respectively. LDL and HDL_3 were further purified by zonal centrifugation as described previously (Patsch et al., 1974). Lipoprotein concentrations were estimated from their phospholipid content which was determined by the procedure of Bartlett (1959). Protein concentrations were determined by the method of Lowry et al. (1951) as modified by Schacterle & Pollack (1973); total and unesterified cholesterol were quantitated enzymatically (Patsch et al., 1976) with the Boehringer-Mannheim kit. Cholesterol was obtained from Sigma Chemical Co. and was recrystallized twice from ethanol. It was judged pure on the basis of its migration as a single spot on SiO_2 plates (Baker) with $CHCl_3$–CH_3OH–H_2O (65:25:4) as visualized with iodine and by its ^{13}C NMR spectrum obtained in $CDCl_3$. Phosphatidylcholine was isolated from hen egg yolks by the procedure of Singleton et al. (1965) and Rouser et al. (1963). Before study by NMR, lipoprotein samples were dialyzed exhaustively against 100 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 1 mM NaN_3, pH 7.4, and were then concentrated in dialysis tubing (1-cm flat width; Spectrapor) by exposure to external dry Sephadex G-150. This procedure caused little or no precipitation or aggregation of the sample as judged by gel filtration on a 0.9 × 100 cm column of Sepharose 2B (Pharmacia). Several samples concentrated by this method gave ^{13}C NMR spectra containing very narrow lines, but these disappeared upon redialysis (for example, see parts A and C of Figure 1).[2] The structural integrity and model lipoproteins were evaluated by gel filtration chromatography before and after NMR measurements. In a representative experiment, a sample of LP-X_t was concentrated to ~150 mg/mL and then subjected to Sepharose 2B gel filtration. Prior to the NMR measurements, an aliquot of this sample eluted as a single peak in a volume of 40–60 mL. After 4 days at 37 °C, the sample exhibited only slight turbidity, and when chromatographed, a major fraction of it (95.3% of the phospholipid) still eluted in the same volume as native LP-X. A small amount (4.7% of the phospholipid) emerged at the void volume of the column (25–35 mL), indicating that a low level of aggregation had occurred.[3] Otherwise, the structural integrity of the lipoprotein appeared to be conserved during the NMR experiment.

Methods. ^{13}C NMR spectra were obtained at natural abundance with a 67.9-MHz spectrometer consisting of a Bruker Model HX-270 superconducting magnet (63.4 kG), a Bruker 10-mm ^{13}C probe, laboratory-built radio-frequency electronics, and a Nicolet 1080 computer. The spectrometer was not equipped for field-frequency lock. Magnetic field stability was checked by measuring the resonance frequency of ethylene glycol before and after each experiment; the drift was 1–3 Hz for all spectra reported herein.

For ^{13}C excitation, 90° pulse widths of 15–17-μs duration were used. Spectra of the unsaturated and glycerocholine region were recorded with the 90° pulse set at a frequency of 13 406.7 Hz downfield from the ^{13}C resonance of Me_4Si. Spectra of the saturated region were obtained with the 90° pulse set 93 Hz downfield from Me_4Si. Time domain spectra were accumulated in 8192 addresses by using a spectral width of 13 888 Hz. Fourier transformations were done on 16 384 time domain points after adding 8192 zeros to each time domain spectrum. To ensure that resonances of interest lay within the envelope of decoupling frequencies, separate spectra of the unsaturated, glycerocholine, and saturated carbon regions were obtained with the ^1H irradiation centered respectively at 5.2, 3.7, and 1.5 ppm downfield from the proton resonance of Me_4Si, by using a random-noise modulation bandwidth of ~500 Hz. The temperature of the sample was measured after equilibration with but removal from the probe. These measurements were done with a Tri-R electronic thermometer and were estimated to be accurate to ±1.5 °C. Line widths ($\nu_{1/2}$) were measured as the peak width at half-height from expanded printouts of the resonances, with an estimated error of ≤15% for resonances of ≤50 Hz width. This error was estimated to increase to ~30% for broader resonances such as cholesterol C6. Chemical shifts were measured with reference to the fatty acyl terminal methyl resonance which appeared 14.1 ppm downfield from tetramethylsilane. Spin–lattice relaxation times (T_1) were measured by the inversion-recovery method (Vold et al., 1968) or the progressive saturation method (Freeman & Hill, 1971; Freeman et al., 1972). Actual T_1 values were determined by an iterative three-parameter fit to the exponential function $A + B^{-\tau/T_1}$ (Kowalewski et al., 1977; Zass & Ziessow, 1977).

The lipids of LP-X were obtained by three to four extractions with $CHCl_3$–CH_3OH (2:1) at room temperature. Phospholipid–cholesterol mixtures and the lipids extracted from LP-X were dispersed in 100 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 1 mM NaN_3, pH 7.4, by using a sonifier cell disrupter (Model W185) from Heat Systems Ultrasound, Plainville, NY. Sonication was performed by using a setting of 7 (35-W power) for 45 min in an ice bath. During sonication, the sample was continuously flushed with zero-grade nitrogen. After sonication, any undispersed lipids and titanium particles were removed by centrifugation in a Beckman Ti 50 rotor at 47 000 rpm for 90 min. Vesicles formed from LP-X lipids eluted from a Sepharose 2B column (0.9 × 100 cm) at a volume comparable to that at which EYPC–cholesterol vesicles eluted.

Results

The chemical composition and structural dynamics of LP-X are reflected in several aspects of its ^{13}C NMR spectrum (Figure 1A). The width of the phospholipid carbonyl resonance is detectably greater for LP-X than for LDL or HDL, a comparison which is also true for several other resonances. This width may be a result of restricted motion for phospholipid carbonyls in LP-X and/or chemical shift heterogeneity arising from the different phospholipid types (Seidel et al., 1970) present in this lipoprotein. The presence of these various types is evident from the spectrum of extracted LP-X lipids in deuteriochloroform (Figure 1B). The two carbonyl resonances of phosphatidylcholine and one of sphingomylein are clustered at 173.1, 173.5, and 173.8 ppm, while the single lysophosphatidylcholine carbonyl appears at 176.5 ppm. Since LP-X_t contains only small amounts of cholesteryl esters (<2%) and triglycerides (<4%), its spectrum does not exhibit the higher field carbonyl resonance at 171.2 ppm previously assigned to these lipids in HDL (Hamilton & Cordes, 1978; Avila et al., 1978) and LDL (Hamilton et al., 1979). The peptide amide carbonyl envelope typically observed in the 175-ppm region of an HDL spectrum is not present in the spectrum of native LP-X_t, consistent with its low abundance of protein (<6%).

The olefin region (120–145 ppm) also shows features indicative of LP-X composition and dynamics. No resonances

[2] In these studies, two very narrow lines frequently appeared in the ^{13}C spectra of LP-X and other aqueous samples. These occurred at 17.3 and 57.9 ppm (indicated by dots in parts A and C of Figure 1) and disappeared upon redialyzing the sample, suggesting that the Sephadex G-150 contained small amounts of low molecular weight material which crossed the dialysis membrane into the sample during concentration.

[3] Data not shown but available upon request.

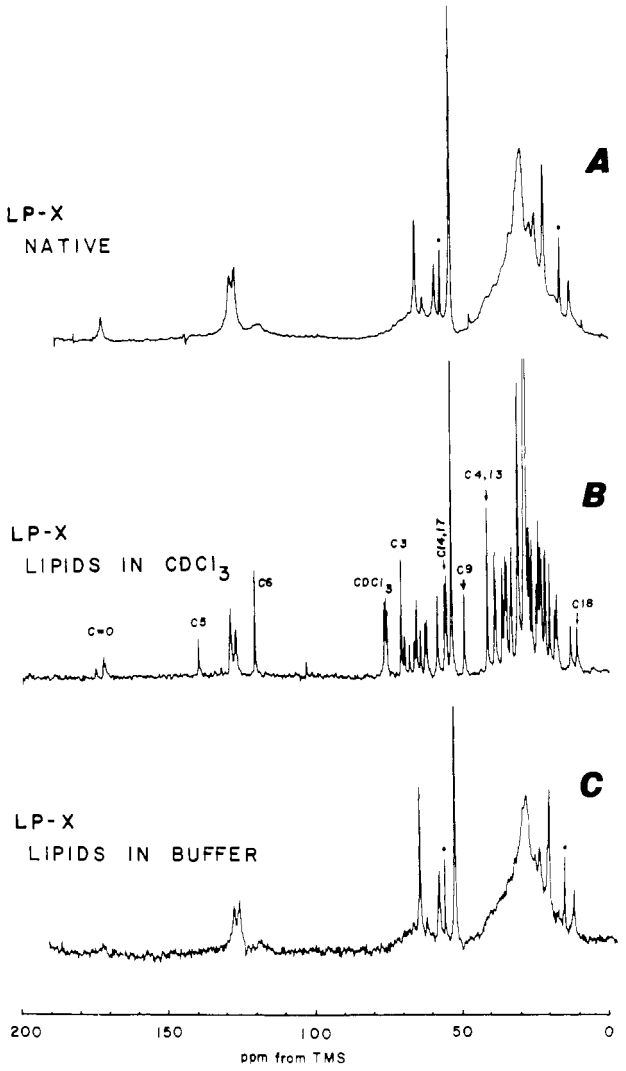

FIGURE 1: 67.9-MHz ^{13}C NMR spectrum of the following. (A) LP-X_t at 151 mg/mL in 100 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 1 mM NaN_3, pH 7.4. Conditions: 65 636 scans; 0.571-s pulse interval; 36 °C. (B) LP-X_t lipids in $CDCl_3$. Lipids were obtained by extraction of LP-X_t with $CHCl_3$–CH_3OH (2:1), followed by evaporation to dryness and redissolution in $CHCl_3$. Conditions: 4096; scans; 3.0-s pulse interval; 31 °C; 78.6 mg of phospholipid/mL; 33.6 mg of cholesterol/mL. (C) LP-X_t lipids in buffer. Extracted lipids were evaporated to dryness in vacuo, then resuspended in 5 mL of 100 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 1 mM NaN_3, pH 7.4, sonicated for 45 min at 0 °C, and then centrifuged at 50 000 rpm in a Beckman 50 Ti rotor at 10 °C. Under these conditions, very little lipid floated to the top or sank to the bottom, indicating high conversion to single bilayer vesicles. Conditions: 32 768 scans; 0.571-s pulse interval; 36 °C; 38.5 mg of phospholipid/mL; 17.9 mg of cholesterol/mL.

for C6 (122.3 ppm) or C5 (139.8 ppm) of esterified cholesterol (Avila et al., 1978) are observed for LP-X whereas these lines are readily apparent in the spectrum of LDL in which cholesteryl esters are much more abundant. The abundance of unesterified cholesterol (~25%) in LP-X is not readily apparent from the spectrum of the native particle; no C5 resonance at 141.3 ppm and only a rather broad C6 line at 120.7 ppm are observed. Possible reasons for the breadth of these resonances are considered below. The envelope containing resonances attributable to the unsaturated carbons of the fatty acyl chains (128–130 ppm) is significantly broader in the LP-X spectra than in the spectra of LDL, HDL, and EYPC vesicles (Figure 1A and parts A and B of Figure 2).

The glycerocholine region (50–80 ppm) contains the choline methyl resonance (54.4 ppm) whose intensity reflects the

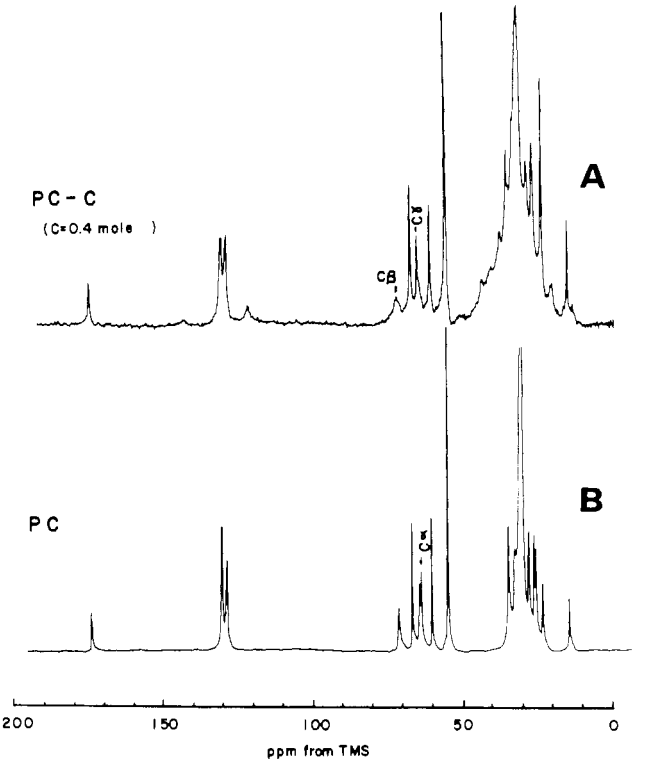

FIGURE 2: 67.9-MHz ^{13}C NMR spectra of the following. (A) Single-bilayer vesicles of egg yolk phosphatidylcholine containing 0.4 mol fraction of cholesterol (0.33 g of cholesterol/g of phospholipid) which is comparable to that in LP-X (0.38 g/g). The lipids were dispersed by sonication, and the vesicles were purified by centrifugation in a Ti-50 rotor at 47 000 rpm for 90 min and chromatography of the resulting supernatant on a 2.5 × 50 cm column of Sepharose 4B. The fractions containing the single-walled vesicles were concentrated by ultrafiltration using an Amicon XM-100 membrane which had been exposed previously to lipid. Conditions: 171 mg of phospholipid/mL; 16 384 scans; 0.571-s pulse interval. (B) Single-bilayer vesicles of egg yolk phosphatidylcholine in the absence of cholesterol. Vesicle preparation and spectrometer conditions were the same as those described above. Phospholipid concentration = 185 mg/mL.

abundance of choline-containing phospholipids in LP-X. The abundance of this lipid is also suggested by the intensities of the CH_2N (66.4 ppm) and CH_2O (59.9 ppm) resonances. In contrast, the phospholipid glyceride carbon resonances are extremely broadened and highly attenuated, compared to those of normal LDL, HDL_3 or cholesterol-containing EYPC vesicles (Figure 2A). The Cγ–Cα doublet which normally occurs at 64.0–63.3 ppm in spectra of phosphatidylcholine vesicles (Figure 2B) is barely discernible in spectra of native (Figure 1A) or partially reconstituted (Figure 1C) LP-X. The sharp Cβ resonance seen at 71.0 ppm in the vesicles (Figure 2B) is broadened so severely in LP-X (Figure 1A) that it appears as a wide shoulder on the low-field side of the CH_2N resonance. This effect can be attributed at least in part to the presence of cholesterol (see Figure 2A).

The aliphatic region (0–50 ppm) of the LP-X spectrum is also distinctly different from the corresponding region of the LDL and HDL spectra. Not only is the major methylene envelope centered at ~30 ppm broadened, but also resonances of specific cholesteryl nuclei such as C4,13 (42.4 ppm), C16,24 (39.8 ppm), C10,22 (36.7 ppm), C19 (19.3 ppm), C21 (18.8 ppm), and C18 (11.9 ppm) which are clearly seen in the LDL and HDL spectra[4] but are not detected in the LP-X_t spectrum (Figure 1A).

[4] These lines are attributable primarily to cholesteryl esters.

Table I: Line Widths of Selected Single Carbon Resonances of Unfractionated LP-X_t, Its Three Subfractions, Its Sonicated Lipid Extract, and Normal HDL and LDL[a]

sample	line width (Hz)								
	CE C6	UC C6	Cβ	CH_2N	Cγ	Cα	CH_2O	$N(CH_3)_3$	fa CH_3
LP-X_1	–	~210	~290	26	28	NO[b]	25	18	25
LP-X_2	–	~160	~280	25	28	NO	23	16	31
LP-X_3	–	~190	~240	26	23	NO	28	18	24
LP-X_t	–	~260	NM[c]	25	25	NO	28	19	38
LP-X_t lipids[d]	–	>350	NM	20	43	NO	22	14	27
LDL	33	–	–	23	–	–	22	13	13
HDL_3	10	–	64	20	40	74	20	13	11
EYPC		–	39	18	26	32	17	18	17
UC-EYPC (0.25 mol/mol)		60	68	20	28	46	20	16	14
UC-EYPC (0.66 mol/mol)		120	115	22	27	75	18	14	16

[a] Values were obtained at 36 °C and are given in hertz. Estimated accuracy is ±30% for cholesterol C6 and Cβ resonances of LP-X samples, ±15% for other resonances of LP-X samples, and ±10% for all resonances of LDL and HDL_3. Reported values *include* 3.2-Hz digital broadening. [b] NO = not observable. [c] NM = not measurable. [d] Dispersed by sonication in 100 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 1 mM NaN_3, pH 7.4. All other samples present are in the same buffer.

Table II: ^{13}C Spin-Lattice Relaxation Times for Selected Nuclei of LP-X and Relevant Model Systems[a]

resonance assignment	chemical shift (ppm)	spin-lattice relaxation times (ms)				
		LP-X_t (a)	LP-X_1 (b)	UC-EYPC, 0.66 mol/mol (c)	UC-EYPC, 0.25 mol/mol (d)	EYPC alone (e)
fa olefin	129.8	662 ± 15	675 ± 25	548 ± 74	654 ± 50	774 ± 44
fa olefin	128.0	881 ± 87	766 ± 60	572 ± 83	698 ± 71	904 ± 48
UC C6	120.1	NM	162 ± 67	119 ± 47	193 ± 18	NP
glyc Cβ	71.0	NM	221 ± 48	224 ± 90	200 ± 5	199 ± 64
chol CH_2N	66.3	335 ± 105	367 ± 33	375 ± 25	320 ± 31	369 ± 16
glyc Cγ	63.9	NM	137 ± 11	89 ± 21	132 ± 4	131 ± 17
glyc Cα	63.3	NM	NM	99 ± 25	133 ± 3	122 ± 37
chol CH_2O	59.8	364 ± 111	372 ± 39	358 ± 45	391 ± 25	356 ± 15
chol $(CH_3)_3N^+$	54.4	525 ± 167	575 ± 68		687 ± 65	

[a] T_1 values for samples a, b, and d were measured by the inversion-recovery method (Vold et al., 1968); for samples c and e, the progressive saturation method was used (Freeman & Hill, 1971; Freeman et al., 1972). T_1 values were obtained by fit of the experimental magnetization amplitudes to the three-parameter exponential function $A + Be^{\tau/T_1}$ (Kowalewski et al., 1977; Zass & Ziessow, 1977). Chemical shifts are given as ppm from Me_4Si and are accurate to ±0.1. The fatty acyl terminal CH_3 resonance was used as an internal reference at 14.1 ppm (Hamilton et al., 1973). NP = not present; NM = not measurable; fa = fatty acyl; UC = unesterified cholesterol; glyc = glyceride; chol = choline.

Many of the above differences observed by inspection are corroborated by line width measurements for selected *single*-carbon resonances (Table I). The line width of the glyceride Cβ resonance for LP-X subfractions ranges between 240 and 290 Hz, ~4 times that observed for the corresponding resonance in HDL_3 and >2 times that for EYPC vesicles containing a comparable proportion of cholesterol. A significant difference in line width is also observed for the fatty acyl terminal CH_3 resonance: 38 Hz in LP-X_t and ~1/3 that width in HDL_3, LDL, and EYPC either with or without cholesterol. In contrast, resonances of carbons in the choline moiety of LP-X (i.e., CH_2N, CH_2O, and $N^+[CH_3]_3$) have line widths comparable to those of LDL and HDL.

The spin–lattice relaxation times (T_1) of selected carbon nuclei in unfractionated LP-X_t, the LP-X_1 subfraction, and phosphatidylcholine single bilayer vesicles containing various amounts of cholesterol are presented in Table II. When the cholesterol/phospholipid molar ratio in EYPC vesicles (Figure 2) is increased 0 → 0.25 → 0.66, a monotonic decrease in T_1 is observed for selected fatty acyl olefin resonances: 774 → 654 → 548 ms for the 129.8-ppm line (monounsaturated) and 904 → 698 → 572 ms for the 128.0-ppm line (polyunsaturated). The T_1 value for the 129.8-ppm line in LP-X_t (662 ms) and LP-X_1 (675 ms) closely approximates that of the model system containing 0.25 mol of cholesterol/mol of phospholipid (654 ms). T_1 for the 128.0-ppm resonance of LP-X_1 (766 ms) also approximates that of the above model system (698 ms). However, the T_1 for this resonance in LP-X_t (881 ms) correlates better with that of the corresponding resonance in pure egg PC vesicles (904 ms). T_1 values for glycerocholine resonances in the various systems ranged from relatively short (89 ms for Cγ) to much longer (687 ms for choline $[CH_3]_3N^+$). The resonances from corresponding carbon nuclei in the glycerocholine region of phospholipids exhibited similar spin–lattice relaxation times in all the systems studied.

The three LP-X subfractions isolated by zonal ultracentrifugation gave spectra which were identical, within experimental uncertainty, as judged qualitatively by their appearance (Figure 3) and quantitatively by the line widths of selected resonances (Table I). However, the spectrum of LP-X_t does differ detectably from those of the LP-X subfractions. The aliphatic (10–40 ppm) regions of the LP-X subfraction spectra (Figure 3) contain several lines which are either attenuated or absent from the unfractionated LP-X_t spectrum (Figure 1A). The glycerocholine region (50–80 ppm) contains a broad but resolved Cβ resonance in the subfraction spectra, but in the LP-X_t spectrum this resonance appears only as a shoulder on the low-field side of the CH_2N line. Possible reasons for these differences are considered below.

The temperature dependence of the LP-X spectrum was studied between 35 and 50 °C, and resonances in two regions of the spectrum responded to this perturbation as shown in Figure 4. In the olefin region, the cholesterol C6 line decreased in width (210 → 170 → 98 Hz) as the temperature was raised (35 → 41 → 50 °C). The glyceride Cβ resonance

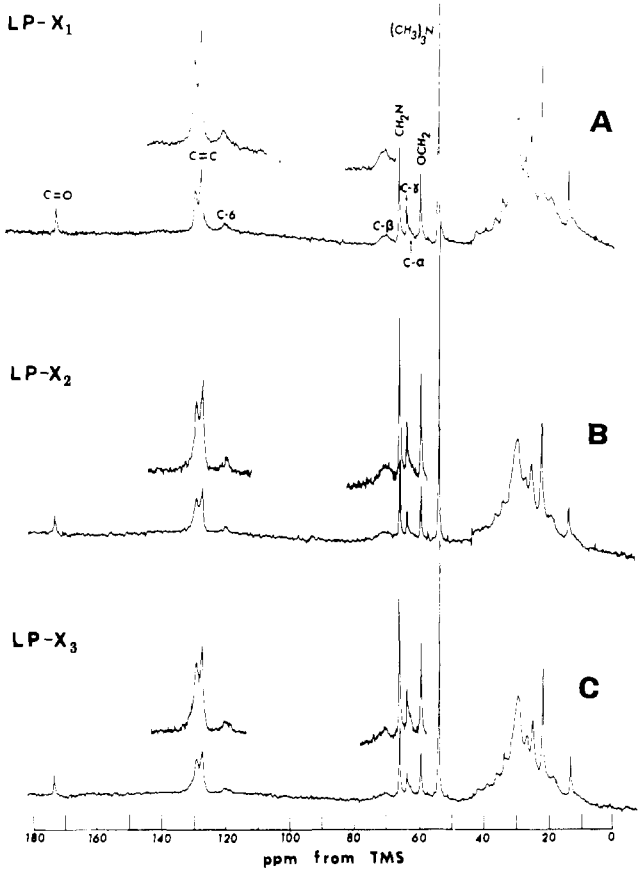

FIGURE 3: 67.9-MHz ^{13}C NMR spectra of LP-X subfractions. (A) LP-X_1, 145 mg/mL; 32 768 scans; 0.571-s pulse interval; 35 °C; (B) LP-X_2, 131 mg/mL; 65 536 scans; 0.571-s pulse interval; 36° C; (C) LP-X_3, 127 mg/mL; 65 536 scans; 0.571-s pulse interval; 35 °C.

remained unchanged with respect to line width (182 → 164 → 173 Hz) but showed a significant increase in apparent intensity at 50 °C. This effect was accompanied by appearance of the ill-resolved glyceride Cα resonance on the high-field side of the Cγ line (Figure 4).

Delipidation of LP-X with $CHCl_3$–CH_3OH (2:1) gives an extract whose spectrum contains all the expected resonances at intensities consistent with their abundances in the native particle (Figure 1B). When these lipids are evaporated to dryness and sonicated in buffer, vesicles are obtained which have a cholesterol/phospholipid ratio similar to that in LP-X (0.35 g/g). Although the spectrum of these vesicles (Figure 1C) is of lower signal/noise, it contains the same general features as those indicated in the higher quality spectrum of LP-X_t (Figure 1A).

Discussion

A characteristic feature of the ^{13}C NMR spectrum of LP-X which clearly distinguishes it from the spectra of normal lipoproteins studied to date is its relatively broad lines. A number of previous investigations have established a relationship between the natural line width (or the spin–spin relaxation time) and the dynamics of lipid fatty acyl chains (Seiter & Chan, 1973; Gent & Prestegard, 1977; Brainard, 1979; Godici & Landsberger, 1975; Lee et al., 1974; Sears, 1975). In systems where the motion is so complicated that it cannot be described by simple models, interpretation of

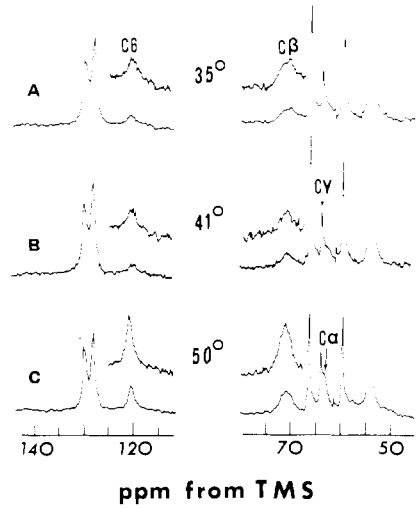

FIGURE 4: 67.9-MHz ^{13}C NMR spectrum (olefin and glycerocholine regions only) of LP-X_1 at (A) 35, (B) 41, and (C) 50 °C. Conditions: 32 768 scans; 0.571-s pulse interval; 145 mg of lipoprotein/mL. An additional spectrum recorded at 35 °C after 50 °C was identical with the orginal 35 °C spectrum.

relaxation times in terms of molecular motions is not straightforward. However, in general, a decreased spin–lattice or spin–spin relaxation time suggests decreased motion, characterized by either a longer correlation time and/or a smaller motional amplitude. Spin–spin relaxation is particularly sensitive to motions occurring at frequencies less than the Larmor frequency, whereas spin–lattice relaxation is more sensitive to motions occurring at or above that frequency.

The large width of LP-X spectral envelopes containing the olefin and aliphatic carbon resonances suggests that the dynamics of the carbon nuclei in the fatty acyl chains of LP-X are significantly different from those in the normal lipoproteins, LDL and HDL. However, contributions to the apparent line widths of these multiresonance envelopes from sources other than those related to dynamics must be carefully considered. The apparent differences in envelope widths could possibly reflect (a) changes in the chemical shifts of incompletely resolved resonances within the envelopes, (b) changes in the relative intensities of resonances within these envelopes, (c) changes in the natural line widths of the individual resonances comprising the envelopes, or (d) a combination of these possibilities. The chemical shifts of resolved resonances in the region of the olefin and aliphatic envelopes are the same, within experimental error, for LP-X, normal lipoproteins, and EYPC–UC vesicles. This suggests it is unlikely there are differences in the chemical shifts of unresolved resonances within the envelopes of such magnitude as to add significantly to differences in the width of the envelopes in these different systems. Furthermore, fatty acid analysis of LP-X and the normal lipoproteins[3] reveals only small differences in their total fatty acid composition, suggesting that changes in the intensities of unresolved resonances arising from heterogeneous fatty acyl chains are not likely to be the cause of the differences in apparent line widths of these envelopes. Therefore, we believe that the differences in the width of these envelopes primarily reflect differences in natural line width of the constituent resonances rather than differences in chemical shift or relative intensity. The observed differences in line widths of LP-X fatty acyl carbon resonances from those of corresponding resonances in normal lipoproteins may originate from two sources. First, the fatty acyl chains of phospholipids in

HDL and LDL may undergo considerably more motion than the corresponding chains in LP-X. Second, the fatty acyl carbon envelopes in spectra of HDL and LDL should have appreciable contributions from acyl chain carbons of cholesteryl esters (21 and 38%, respectively) and triglycerides (14 and 25%, respectively), lipid molecules which by reason of their predominantly hydrophobic and nonamphiphilic character may be expected to assume locations and to undergo motions different from those of phospholipids in the same particle. Unfortunately, it is not possible to separate the contributions from each of these lipid types. However, meaningful comparisons can be drawn between spectra of LP-X and spectra of sonicated EYPC vesicles, since for both systems the majority of the fatty acyl chains are contained in phospholipids. Comparison of the spectra of LP-X, EYPC vesicles alone, and EYPC vesicles with cholesterol reveals that the olefin and aliphatic envelope line widths increase with increasing cholesterol/phospholipid ratio (Table I), which can be attributed chiefly to the rigidifying effect of cholesterol on fluid liquid-crystalline phospholipid bilayers (Lee et al., 1974, and references cited therein).

Comparison of the single carbon resonances detectable in spectra of LP-X, HDL, LDL, and sonicated vesicles which may be unambiguously assigned to carbon nuclei within a single lipid class (e.g., the choline methyl and methylene resonances and the Cα glyceride resonance which arise only from phospholipid carbons) reveals that their line widths are remarkably similar in all the systems studied. In addition, the spin–lattice relaxation times of the choline methyl and methylene carbons are the same, within experimental error. These observations suggest that the dynamics of the headgroup region of phospholipids are quite similar in these systems, at least for those motions which are reflected in the ^{13}C relaxation times. It has been shown previously that the dynamics and orientation of certain nuclei in the polar head groups of phospholipid bilayers are not very sensitive to the presence of cholesterol (Godici & Landsberger, 1975; Brainard, 1979; Brown & Seelig, 1978; Yeagle et al., 1975). In contrast, it has been demonstrated (Brainard, 1979; J. R. Brainard and E. H. Cordes, unpublished experiments) that the presence of cholesterol in sonicated vesicles markedly affects the line widths of the single-carbon resonances arising from the Cα and Cβ glyceride carbons. This same behavior is seen for these resonances in spectra of LP-X. The Cβ glyceride resonance in spectra of LP-X is so broad in several spectra that it is not resolved from the broad base-line rise which occurs in the glycerocholine region of the spectrum (Figure 1A). The Cα carbon resonance is never resolved from the neighboring Cγ resonance. These observations suggest that the motion of carbons in the glyceride moiety of phospholipids is substantially more restricted in LP-X than in any of the other systems studied. In addition, the line width of the unesterified cholesterol C6 resonance is considerably broader in spectra of LP-X than of HDL (10–20 Hz); it is also broader than the esterified cholesterol C6 resonance in HDL (10 Hz) or LDL (33 Hz). Hence, these data suggest that the motions of unesterified cholesterol within the LP-X particle are considerably restricted relative to the motions of cholesterol and cholesteryl esters in normal lipoproteins.

In an effort to more clearly understand the dynamics of cholesterol within the LP-X particle, we have computed the line width of the cholesterol C6 resonance for two limiting models of cholesterol motion. Both models are based on the assumption that the dominant relaxation mechanism for this nucleus is dipolar. The first model assumes that cholesterol

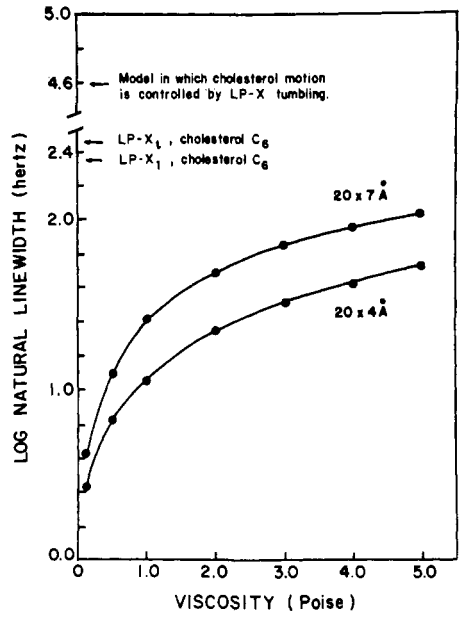

FIGURE 5: Plot of log of the natural line width vs. viscosity for a methine carbon nucleus undergoing dipolar relaxation at 63 kG with a proton at distance of 1.09 Å. The C–H internuclear vector is assumed to be attached to an ellipse of dimensions 20 × 7 or 20 × 4 Å undergoing rotational diffusion at 30 °C in isotropic media of varying viscosity. The dimensions of the ellipse were selected to represent the size of a cholesterol molecule, and the C–H internuclear vector was inclined at 60° to the major axis of the ellipse, approximating the position of the C6–H internuclear vector of cholesterol. The arrows indicate the experimentally observed line widths for cholesterol C6 of LP-X_1 and LP-X_t, as well as the C6 line width predicted by a model in which the cholesterol molecule is completely immobile (on the NMR time scale) within the LP-X particle.

cannot undergo any motion within the LP-X particle (i.e., that the correlation time for the cholesterol molecule is the same as that of the entire particle). Using a particle radius of 339 Å (Patsch et al., 1977a), the viscosity of water (0.0078 P), the Stokes–Einstein relationship (Stokes, 1856; Einstein, 1908) for determining the correlation time, and the expressions of Doddrell et al. (1972) for calculating the relaxation times for methine carbon nuclei (r = 1.09 Å), we predict a value of ~41 000 Hz for the natural line width of the C6 resonance. The observed line widths are vastly smaller, suggesting that the cholesterol molecule undergoes considerable motion independent of the LP-X particle. In the second model, the cholesterol molecule undergoes unrestricted rotational diffusion in an isotropic medium with a viscosity similar to that found in the hydrophobic interior of phospholipid–cholesterol bilayers. For these calculations, we have assumed that the shape of the cholesterol molecule may be approximated by a prolate ellipsoid whose motion within the bilayer may be predicted by Perrin's equations for rotational diffusion (Perrin, 1934; Woessner, 1962). The best estimates for the viscosity within these bilayers derived from fluorescent probe measurements are in the range 2–5 P at ~30 °C (Jonas, 1977; Shintisky & Inbar, 1974). It should be borne in mind that these estimates undoubtably reflect the anisotropic nature of the bilayer interior. An alternative estimate for the viscosity of this hypothetical "isotropic" hydrocarbon interior would be a value similar to that measured by conventional rheological techniques for liquid hydrocarbons. Typical viscosities for these systems range from 0.033 P (n-hexadecane at 20 °C) to 0.84 P (olive oil at 20 °C) (Weast, 1975). The range of natural line widths predicted for a methine carbon nucleus attached to an ellipsoid similar in size to a cholesterol molecule undergoing rotational diffusion in an isotropic medium of viscosity 0.1–5.0 P is shown

in Figure 5. The dimensions 4 × 20 and 7 × 20 Å were selected to represent the minimum and maximum dimensions of an ellipsoid body similar in size and shape to a cholesterol molecule. Also shown are the observed line widths for native LP-X and the value predicted for a cholesterol molecule completely immobilized within the LP-X particle. The experimentally observed line widths (210–280 Hz) are 2–10 times greater than those predicted for a cholesterol molecule freely diffusing in an isotropic medium (<100 Hz) (Table I and Figure 5); however, these experimental values are only ~1/500 of that predicted for a cholesterol molecule completely immobilized within an LP-X particle (~41 000 Hz). These comparisons indicate that the motion of unesterified cholesterol in LP-X is between these two extreme models.

For sonicated EYPC–UC vesicles, the T_1 values for the fatty acyl olefin envelopes and for the cholesterol C6 resonance decrease with increasing mole fraction of cholesterol. Other investigators have also noted a small decrease in the spin–lattice relaxation times for olefin resonances in sonicated EYPC–UC vesicles (Godici & Landsberger, 1975). In the present study, the observed changes are slightly larger than the experimental uncertainty in the values, but it is difficult to establish the magnitude of the effect of cholesterol upon the segmental motion in these systems. Further studies are required to reduce the experimental uncertainties in the spin–lattice relaxation times to levels permitting more reliable comparison of trends which are presently more suggestive than definitive. Since the T_1 values for the fatty acyl olefin resonances of LP-X fall in the range observed for sonicated EYPC–UC vesicles, they suggest that the segmental mobilities of the fatty acyl chains of the phospholipids in these two systems are quite similar.

There are qualitative differences between the spectra of total LP-X and the subfractions. The resolution of the Cβ phospholipid glyceride carbon and the cholesterol resonances lying on the high-field side of the fatty acyl methylene resonances is considerably enhanced in the spectra of the three subfractions (Figure 3). The heterogeneity in size and chemical composition of the unfractionated mixture could result in a chemical shift heterogeneity of the resonances, leading to a reduction in their resolution in the resulting spectrum. However, the chemical shifts of the resonances of the various subfractions are the same within experimental error. The observation of a resolved Cβ phospholipid glyceride resonance and the apparently narrower resonances in the fatty acyl methylene envelope suggest that these groups may experience more motional freedom in the LP-X subfractions. The apparent spectral differences may reflect differences in particles from different donors and/or differences resulting from physical changes resulting from the additional zonal centrifugation required to isolate the purified subfractions.

The ^{13}C NMR spectra show only slight changes with increasing temperature (Figure 4), in agreement with earlier EPR data (Patsch et al., 1977a). However, two of the broader resonances, phospholipid glycero Cβ and cholesterol C6, increase in intensity as the temperature is increased. This intensity increase could possibly be due to a change in the T_1 of the resonance, to an increased nuclear Overhauser effect associated with the carbon resonance, or to greater numbers of phospholipid and cholesterol molecules which are contributing to the detectable NMR signals. The last explanation, if correct, suggests that a portion of the phospholipid and cholesterol within LP-X may be organized so as to yield an NMR signal which is undetectable under our experimental conditions. In addition, the temperature dependence suggests that the equilibrium between molecule populations yielding unobservable and observable NMR signals is affected by temperature.

Our ^{13}C NMR results suggest a different picture of the comparative fluidities of LP-X with respect to the normal lipoproteins and phospholipid vesicles than that resulting from fluorescent probe measurements obtained with DPH (Jonas, 1977). They indicate that at ~37 °C, the fluidity of LP-X is considerably less than that of normal LDL or HDL. This interpretation is based primarily upon the comparative line widths of ^{13}C NMR resonances and is in agreement with other results obtained from EPR (Patsch et al., 1977a) and ^{1}H NMR studies [see Brainard et al. (1980)]. In contrast, the microviscosity of these systems as measured by DPH suggests that LP-X is *significantly more* fluid than EYPC–UC vesicles and *slightly more* fluid than HDL and LDL. The difference between our interpretation and that derived from the fluorescence measurements may be related to the position and distribution of the fluorescent probe within the lipid systems studied and in the treatment of fluorescent depolarization data derived from systems in which the probe's motion is considerably hindered (Prendergast & Lakowicz, 1979).

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Lipoprotein-X: Proton and Phosphorus-31 Nuclear Magnetic Resonance Studies on Native, Reconstituted, and Model Systems†

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ABSTRACT: LP-X, a lipoprotein present in the low-density range (*d* 1.006-1.063 g/mL) of cholestatic human plasma, has been studied with its normal counterpart (LDL) by ^1H and ^{31}P nuclear magnetic resonance. The 220-MHz ^1H spectrum of LP-X contains four major lines: the choline CH_2N and $N^+(CH_3)_3$ resonances and the cholesteryl-acyl CH_2 and CH_3 envelopes. The widths of these four lines at 37 °C are approximately 24, 10, 124, and 48 Hz, respectively. The latter two line widths are much greater than the corresponding ones of LDL (28 and 20 Hz), suggesting the much more restricted motion of acyl chains and/or cholesteryl rings in LP-X. This difference persists over the temperature range 15-52.5 °C. The microscopic fluidity of LP-X and LDL was compared by titration with 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo), a paramagnetic amphiphile which distributes between the bulk aqueous phase and the fluid lipid phase of lipoproteins. Tempo is much less effective in broadening the ^1H resonances of LP-X than of LDL, indicating the lower permeability/fluidity of the former. The 40.5-MHz ^{31}P spectrum of LP-X consists of a single resonance whose line width is ~20 Hz and whose spin-lattice relaxation time is 2.23 ± 0.15 s. Titration of LP-X with Pr^{3+} ions splits this resonance into two lines, one remaining at the chemical shift of the original resonance and the other paramagnetically shifting downfield. The ratio of integrated areas for these two lines was 1:1.72. Titration of phosphatidylcholine–cholesterol vesicles alone, vesicles containing apolipoprotein-C and albumin, or vesicles containing apolipoprotein-X gave results similar to those obtained with native LP-X, suggesting the presence of a single bilayer structure in all of these systems.

Subjects suffering from cholestasis or lecithin:cholesterol acyltransferase deficiency frequently have elevated blood levels of unesterified cholesterol which is transported principally in the form of a lipoprotein known as LP-X.[1] The chief components of this lipoprotein are phospholipid (~65%) and cholesterol (~25%). Previous reports from this laboratory (Patsch et al., 1977) and others (Hauser et al., 1977; Laggner et al., 1977) have provided good evidence that LP-X posesses structural features which qualify it as a bilamellar vesicle. This structural property distinguishes LP-X from all other mature circulating lipoproteins, which can be described as micelles with an outer monolayer shell of phospholipid, protein, and some but perhaps not all of the unesterified cholesterol and with an inner core of cholesteryl ester and triglyceride (Morrisett et al., 1977). Another physical property which distinguishes LP-X from normal lipoproteins is its relatively low level of fluidity (Patsch et al., 1977). In the present study, these two physical properties, vesicular structure and micro-

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[1] Abbreviations used: NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; HSA, human serum albumin; apoC, the mixture of apoproteins obtained from human very low density lipoproteins after delipidation and removal of apoB by gel filtration over Sephadex G-150; EDTA, ethylenediaminetetraacetate; VLDL (IDL, LDL, and HDL), very low (intermediate, low, and high) density lipoproteins typically isolated ultracentrifugally at *d* <1.006 (1.006-1.019, 1.019-1.063, and 1.063-1.210) g/mL; EYPC, egg yolk phosphatidylcholine; T_1, spin-lattice relaxation time; UC, unesterified cholesterol; *d*, density in g/mL.

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