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

James R. Brainard,‡ Eugene H. Cordes,§ Antonio M. Gotto, Jr., Josef R. Patsch, and Joel D. Morrisett*

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-

† From the Department of Chemistry, Indiana University, Bloomington, Indiana 47401 (J.R.B. and E.H.C.), and the Department of Medicine, Baylor College of Medicine, The Methodist Hospital, Houston, Texas 77030 (A.M.G., J.R.P., and J.D.M.). *Received September 20, 1979; revised manuscript received April 30, 1980.* This research was supported by a National Research and Demonstration Center grant (HL 17269-03) from the National Heart, Lung, and Blood Institute, a grant-in-aid from the American Heart Association (78-1042) to J.D.M., and a grant from the U.S. Public Health Service (6M 19631) to E.H.C.

* Correspondence should be addressed to this author. He is an Established Investigator of the American Heart Association.
‡ Present address: Baylor College of Medicine, The Methodist Hospital, Houston, TX 77030.
§ Present address: Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065.

[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.

0006-2960/80/0419-4273$01.00/0

scopic fluidity, are examined in further detail by ^1H and ^{31}P NMR.

Experimental Section

Materials. LP-X and its three subfractions were prepared by Cohn fractionation and zonal ultracentrifugation on a linear NaBr gradient (*d* 1.00–1.15) as described by Patsch et al. (1977). Lipoproteins were delipidated by ether–ethanol (3:1) extraction. LDL was obtained from normal plasma by ultracentrifugation in a Beckman 50.2 Ti rotor at 50 000 rpm, 10 °C, for 18 h. After removal of contaminating VLDL and IDL by an initial centrifugation at *d* 1.019, a second spin at *d* 1.063 was used to float the desired LDL. Total HDL was subsequently isolated at *d* 1.210. Lipoprotein concentrations were estimated by either their phosphorus content (LP-X, HDL, and LDL) as determined by the method of Bartlett (1959) or their protein content (HDL and LDL) as determined by the method of Lowry et al. (1951). Cholesterol was obtained from Sigma Chemical Co. and recrystallized twice from ethanol. It was considered to be >99% pure on the basis of its thin-layer chromatographic behavior and its ^{13}C NMR spectrum obtained in $CDCl_3$. Cholesterol was quantitated by an enzymatic method (Patsch et al., 1976). Phosphatidylcholine was prepared from hen egg yolks by the procedure of Singleton et al. (1965) and Rouser et al. (1963).

2,2,6,6-Tetramethylpiperidinyl-1-oxy (Tempo) was prepared by the method of Rozantsev (1970) and was purified by vacuum distillation at 22 °C. The distillate solidified in the receiver at 0 °C. $Pr(NO_3)_3 \cdot 6H_2O$ was obtained from the Alpha Division of Ventron Corp.

Phospholipid–cholesterol mixtures were dispersed by sonicating for 30–45 min in 100 mM NaCl, 10 mM Tris, 1 mM EDTA, and 1 mM NaN_3, pH 7.4. The sonication was typically performed on a 3-mL sample cooled in an ice bath and continuously flushed with nitrogen. Sonicates were then centrifuged at 47 000 rpm in a Beckman 50 Ti type rotor. The center third of the volume consisted almost exclusively of single-bilayer vesicles as demonstrated by gel filtration chromatography (data not shown) (Barenholz et al., 1977) and was used for these studies.

Model systems containing apoC and human serum albumin were prepared by incubating sonicated EYPC–UC vesicles with appropriate aliquots of solutions of apoC and HSA at concentrations of about 5 and 7 mg/mL, respectively. The complexes were isolated on a Sepharose 4B gel filtration column (2.8 × 50 cm). Subsequent gel filtration on an analytical Sepharose 2B gel filtration column (1.2 × 90 cm) demonstrated that >99% of the protein and lipid were associated in a single complex eluting at a volume identical with that for pure EYPC–UC vesicles.

Methods. After isolation and purification, lipoproteins were concentrated in Spectrapor dialysis tubing (1-cm flat width) by exposure to external Sephadex G-150. Samples for ^{31}P NMR experiments were dialyzed exhaustively in 100 mM NaCl, 10 mM Tris, 1 mM EDTA, and 1 mM NaN_3, pH 7.4. Samples for ^1H studies were dialyzed vs. D_2O containing 100 mM NaCl, 10 mM sodium phosphate, and 1 mM NaN_3, pD 7.4 (pD = meter reading + 0.4; Glasoe & Long, 1960). ^{31}P experiments were performed on a Varian XL-100-15 NMR spectrometer operating at 23.4 kG (40.5 MHz) and equipped with a Nicolet TT-100 32K data system. Resonances were continuously proton-decoupled except for area integration measurements where gated decoupling was used. Conditions of spectral accumulation and processing are given in the figure and table legends. Samples (2.5 mL) were studied in 12-mm tubes. Chemical shifts are relative to external 85% H_3PO_4.

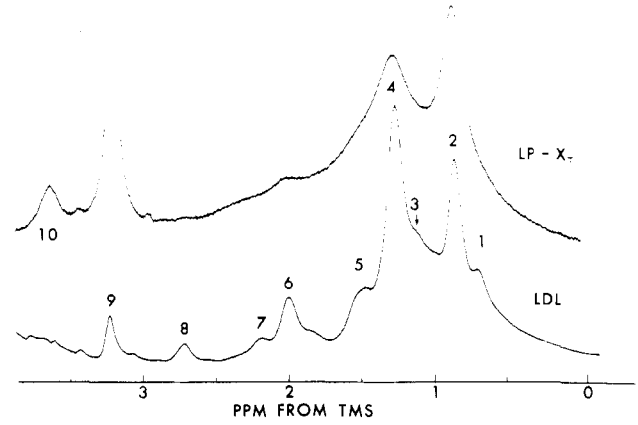

FIGURE 1: 220-MHz ^1H NMR (single scan) of unfractionated lipoprotein-X (LP-X_t; 48.6 mg/mL) and normal low-density lipoprotein (LDL; 45.3 mg/mL) at 37 °C. Chemical shifts, line widths, and assignments for the numbered resonances are presented in Table I. Samples (1 mL) were dialyzed 4 times against 5 mL of D_2O containing 100 mM NaCl, 10 mM phosphate, 1 mM NaN_3, and 1 mM EDTA, pD 7.4.

^1H experiments were performed on a Varian HR-220 NMR spectrometer operating at 51.67 kG (220 MHz). Samples (0.5 mL) were studied in 5-mm precision bore tubes (Wilmad). In some samples, a concentric capillary of tetramethylsilane was used as a chemical shift reference. Temperature was governed with a Varian variable-temperature controller and was measured by the ethylene glycol chemical shift method. Samples were allowed to equilibrate thermally 10 min before their spectra were recorded. Proton resonance assignments were made on the basis of those reported by Finer et al. (1971) and Lee et al. (1974). ^{31}P line widths were measured at half-height from expanded scale printouts of the resonance and are reported in hertz. ^1H line widths were measured directly from the continuous wave spectrum (1000 Hz/50 cm).

Results

The ^1H spectrum of unfractionated LP-X is relatively simple, consisting of only four prominent lines[2] (Figure 1). A characteristic feature of the spectrum is its intense choline methyl resonance (line 9) which is due to the high proportion of phospholipid (~65%) which is reflected to a somewhat lesser extent by the choline CH_2N resonance (line 10), usually undetectable in LDL spectra. Lines 2 and 4 are broad nuclear envelopes which contain resonances originating from cholesteryl and fatty acyl methyl and methylene groups, respectively. Several of the proton types present in both LP-X and LDL are observed in only the spectrum of LDL (Figure 1). Lines 1 and 3 arising from the cholesteryl C18 and C19 methyl protons appear in the LDL spectrum as discernible shoulders, whereas there is no hint of their resolution in the LP-X spectrum. This lack of resolution for these specific resonances of LP-X is accompanied by a similar lack of resolution for nuclei within the fatty acyl chains. For example, the fatty acyl β-methylene (line 5) and the allylic methylenes adjacent to monounsaturated (line 6) and polyunsaturated (line 8) carbons are barely, if at all, detectable in the LP-X spectrum whereas

[2] The word *resonance* has been reserved for that portion of a spectrum attributable to one nuclear type, while *envelope* has been used to describe two or more nuclear types, and *line* has been used to describe either a resonance or an envelope.

Table I: 220-MHz ^{1}H NMR Spectral Data for LP-X_t and LDL at 36 °C

	line	chemical shift (ppm)	line width (Hz)	assignment
LP-X_t	2	0.86	48	cholesterol C21, -26, -27; fa CH_3 [b]
	4	1.26	124	fa CH_2; cholesterol CH_2 [b]
	9	3.22	10	choline $N^+(CH_3)_3$
	10	3.64	24	choline CH_2N
LDL	1	0.64	NM[a]	cholesterol C18
	2	0.84	20	cholesterol C21, -26, -27; fa CH_3 [b]
	3	0.97	NM	cholesterol C19
	4	1.25	28	fa CH_2; cholesterol CH_2 [b]
	5	1.46	NM	fa $CH_2-C-C(=O)$
	6	1.98	34	fa $CH_2-C=C-CH_2$
	7	2.12	NM	fa $CH_2-C(=O)$
	8	2.67	24	fa $CH_2-C=C-C=C-CH_2$
	9	3.20	9	choline $N^+(CH_3)_3$
	10	3.64	12	choline CH_2N

[a] NM = not measurable at 36 °C due to insufficient resolution. [b] Experiments of Kroon et al. (1975) using perdeuterated dipalmitoylphosphatidylcholine vesicles containing undeuterated cholesterol strongly suggest that the resonance for cholesterol C26 and C27 and fatty acyl CH_3 each makes a significant contribution to line 2 but that line 4 consists predominantly of fatty acyl CH_2 resonances.

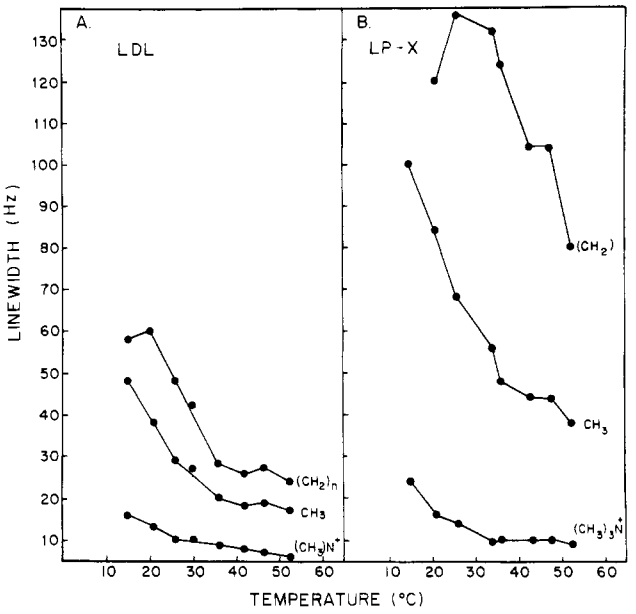

FIGURE 2: Temperature dependence of 220-MHz ^{1}H NMR line widths of LP-X_t and normal LDL. Sample concentrations and preparation are the same as those described in the legend of Figure 1.

these resonances are readily identifiable in the spectrum of LDL (Table I).

These differences in resolution and width of lines observed in the proton spectra of LP-X and LDL not only reflect their different lipid compositions but also suggest fundamentally different structural organization for these two particles. Hence, several different types of experiments were performed to evaluate these possible structural differences. The first set of experiments was designed to determine the relative thermal stability of the two particles. This was achieved by comparing the temperature dependence of corresponding line widths that could be measured, i.e., lines 2, 4, and 9. At 15 °C, the width of the choline methyl resonance (line 9) is 16 Hz for LDL and 24 Hz for LP-X (Figure 2). With increasing temperature, these lines become somewhat narrower; at 52.5 °C, the line width is 9 Hz for LP-X and 6 Hz for LDL. Significantly larger differences were observed in the widths of the methylene

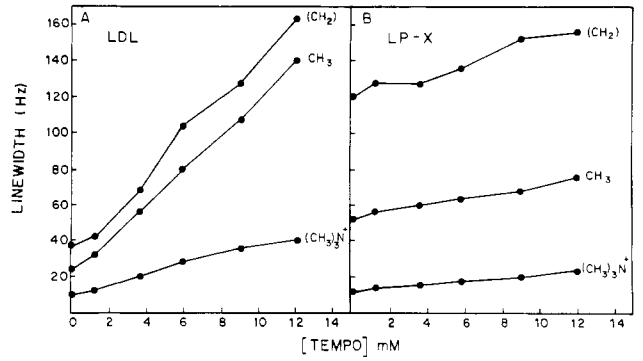

FIGURE 3: Dependence of 200-MHz ^{1}H NMR line widths of (A) LDL and (B) LP-X_t on the concentration of the amphiphilic spin-label 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo). The samples (1 mL each) were dialyzed 4 times against 5 mL of D_2O containing 100 mM NaCl, 10 mM phosphate, 1 mM NaN_3, and 1 mM EDTA, pH 7.4, giving final lipoprotein (lipid) concentrations of 32.7 (31.1) mg/mL for LP-X_t and 45.3 (36.2) mg/mL for LDL. Volumes of 0.5 mL were titrated with aliquots of a 100 mM stock solution of Tempo in D_2O. Spectra were recorded at 37 °C.

envelopes (line 4) for these two lipoproteins over the entire temperature range studied. At 26 °C, this line exhibited a width of 136 Hz for LP-X and 48 Hz for LDL. This differential of 88 Hz decreased to only 56 Hz at 52.5 °C. Similar behavior was observed for the methyl envelope (line 2) as well.

The next set of experiments was designed to determine the relative microscopic fluidity of LDL and LP-X. This was approached through the use of Tempo, a small paramagnetic, amphiphilic molecule which is soluble in aqueous and fluid lipid phases (Shimshick & McConnell, 1972) but is largely excluded from solid, rigid lipid phases. This paramagnetic molecule causes a distance-dependent broadening of proton resonances (Wien et al., 1972; Morrisett et al., 1973b). If Tempo penetrates into the lipid phase, protons on molecules in that phase will experience enhanced nuclear relaxation resulting in an increase in their line widths (Sillerud & Barnett, 1977). Conversely, if Tempo is excluded from a rigid lipid phase, the relaxation of protons on those lipids will be minimally perturbed and their resonances will remain largely unaffected. Titration of LDL with Tempo causes a concentration-dependent increase in the widths of choline CH_3, cholesteryl–acyl CH_3, and cholesteryl–acyl CH_2 lines (Figure 3A). Increasing the Tempo concentration from 0 to 12.1 mM caused a 31-, 116-, and 128-Hz increase in the widths of these respective lines in LDL. For LP-X, the increase in widths of these lines was much smaller: 12 Hz for choline CH_3, 24 Hz for cholesteryl–acyl CH_3, and 30 Hz for cholesteryl–acyl CH_2 (Figure 3B). These results indicate that LDL is significantly more permeable to Tempo at 37 °C than is LP-X.

The steric accessibility of phospholipids within native and model LP-X was studied by ^{31}P NMR. The 40.5-MHz ^{31}P spectrum of LP-X_t consists of a single line with a width of 20 Hz (Figure 4A). This resonance is due primarily to phosphatidylcholine which is reported to comprise 77.5% of the LP-X phospholipids (Seidel et al., 1970). The remaining 4.1% lysophosphatidylcholine, 14.2% sphingomyelin, and 2.5% phosphatidylserine do not appear as resolved resonances probably because of their low abundance and/or restricted motion. In the normal lipoproteins, good resolution between sphingomyelin, lysophosphatidylcholine, and phosphatidylcholine ^{31}P resonances has been observed (Glonek et al., 1974; Henderson et al., 1975). The single phosphorus resonance of LP-X which occurs 31 Hz upfield from external H_3PO_4 splits into two resonances upon addition of Pr^{3+} ions (Figure 4A–E). Upon addition of 1.6 mM Pr^{3+}, a new resonance 68 Hz

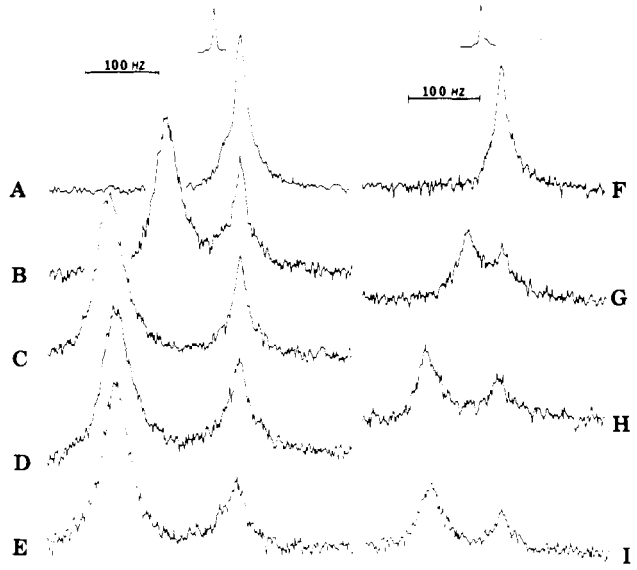

FIGURE 4: 40.5-MHz ^{31}P NMR spectra of native and model lipoprotein-X in the presence of increasing concentrations of Pr^{3+}. Left (A–E): native LP-X_1 at 27.3 mg in 2.5 mL of 100 mM NaCl, 10 mM Tris, 1 mM NaN_3, and 1 mM EDTA, pH 7.4, was titrated with 17.8 mM $Pr(NO_3)_3$ at 34 °C. Conditions: (A) 0 mM Pr^{3+}; (B) 1.6 mM Pr^{3+}; (C) 2.2 mM Pr^{3+}; (D and E) 2.2 mM Pr^{3+}, recorded (D) after 4 days at 40 °C and (E) after an additional 1 h at 55 °C. An external reference signal of 85% H_3PO_4 is shown at the top of the figure. Spectra are from 1024 scans obtained at 1.21-s recycle times. Right (F–I): model LP-X consisting of phosphatidylcholine–cholesterol–human serum albumin–apolipoprotein C. Phosphatidylcholine–cholesterol (2.3 g/g) vesicles were incubated with an albumin–apoC (1:1 g/g) mixture at 37 °C for 1 h and then chromatographed on Sepharose 4B (1.5 × 30 cm column) equilibrated with 100 mM NaCl, 10 mM Tris, 1 mM NaN_3, and 1 mM EDTA, pH 7.4. The eluted model lipoprotein was concentrated to 7.1 mg of phospholipid/mL. A 2.5-mL aliquot was titrated at 25 °C with a 17.8 mM stock solution of $Pr(NO_3)_3$. Pr^{3+} concentrations were (F) 0, (G) 0.63, (H) 0.95, and (I) 0.95 mM.

downfield from H_3PO_4 is observed. Separation of this paramagnetically shifted resonance from the original resonance increases with increasing concentration of Pr^{3+}. At a concentration of 2.2 mM, the two resonances are base-line resolved and separated by 182 Hz (Figure 4C).

The stability of LP-X structure in preserving the accessible and inaccessible environments of the phosphate groups is indicated by the retention of the two resolved resonances, even after incubation of the system for 4 days at 40 °C (Figure 4D) and an additional 1 h at 55 °C (Figure 4E).

One line of evidence supporting the vesicular structure of LP-X is the similar Pr^{3+} titration behavior of well-characterized phosphatidylcholine vesicles containing the same amount of cholesterol as LP-X.[3] This system was titrated over a range of Pr^{3+} concentrations in order to precisely determine that concentration at which the resonances corresponding to the accessible (outside) and inaccessible (inside) phosphate groups become resolved. Detectable separation between two maxima was observed at 0.68 mM Pr^{3+}. By 1.0 mM Pr^{3+}, both resonances were sufficiently resolved to permit measurement of reasonably accurate line widths. The effect of this concentration of Pr^{3+} on the accessible phosphorus nuclei is not only to chemically shift their resonance downfield by 90 Hz but also to broaden that resonance by ~10 Hz. The paramagnetic broadening effect is not felt by the inaccessible nuclei. The effect of Pr^{3+} ions was also studied with phosphatidylcholine–cholesterol vesicles to which human serum albumin and apolipoprotein-C (Figure 4F) or apoLP-X[3] had been bound. The latter two systems had chemical compositions closely resembling that of LP-X (Table II). The single line observed in the absence of Pr^{3+} (Figure 4F) was partially resolved into two resonances at 0.63 mM Pr^{3+} (Figure 4G). These two resonances became completely resolved at 0.95 mM Pr^{3+} without any significant change in the spectrum over a 3-h period (Figure 4I). These results and similar results[3] with apoLP-X indicate that although apoC and albumin or apoLP-X was bound to the phosphatidylcholine–cholesterol vesicles as determined by gel filtration, these proteins did not render the vesicles permeable to Pr^{3+} ions.

[3] Data not shown but available on request.

Table II: Chemical Composition of Representative Samples of Native and Model LP-X

sample	[PL][a]	[UC][b]	[protein][c]	UC/PL	protein/PL
native LP-X_1	25.6	9.6	1.32	0.38	0.05
EYPC–UC vesicles	16.9	5.1		0.30	
EYPC–UC vesicles plus apoLP-X	18.45	7.05	0.23	0.38	0.01
EYPC–UC vesicles plus apoC plus HSA	18.30	7.95	1.73	0.43	0.09

[a] Phospholipid concentration in mg/mL. [b] Unesterified cholesterol concentration in mg/mL. [c] Protein concentration in the mixture in mg/mL.

Table III: Distribution of Phosphorus-Containing Lipids in LP-X and Relevant Model Systems between Sites Accessible (A) and Inaccessible (I) to Pr^{3+} [a]

sample	[PL] (mg/mL)	[Pr^{3+}] (mM)	resonance area ratio (A/I)	calcd surface area ratio (out/in)
LP-X_1	17.0	2.57	1.72	1.38
LP-X_t	15.0	2.06	1.82	1.38[b]
EYPC (no. 1)	21.5	1.71	2.02	2.09
EYPC (no. 2)	20.0	2.56	2.08	2.09

[a] Phospholipid-containing particles in 100 mM NaCl, 10 mM Tris, 1 mM EDTA, and 1 mM NaN_3, pH 7.4, were titrated with $Pr(NO_3)_3$ until the original single phosphorus resonance was sufficiently resolved into two resonances to allow them to be digitally integrated separately. The spectra were obtained at 36 °C under gated decoupling conditions from 2048 free induction decays at 1.21-s pulse intervals. [b] This value was computed from a weighted average of LP-X subfractions, i.e., three LP-X_1 (339-Å radius), two LP-X_2 (343-Å radius), and one LP-X_3 (294-Å radius).

The relative populations of accessible and inaccessible phosphate groups were determined by measuring the integrated intensities of the shifted and unshifted ^{31}P resonances in spectra of LP-X and EYPC vesicles (Table III). These ratios are compared with the outer/inner surface area ratios calculated from the physical dimensions of the particles and the bilayer thickness. For LP-X, the ratios of accessible to inaccessible phosphate groups are *somewhat above* the surface area ratios. In contrast, the ratios of accessible to inaccessible phosphate are *nearly equal* to the outer/inner surface area ratios for pure EYPC vesicles.

^{31}P spectra of LP-X resemble those of phosphatidylcholine–cholesterol vesicle systems not only with respect to width and separation of the phosphorus resonances in the presence and absence of Pr^{3+} ions but also with respect to spin–lattice relaxation times (T_1) (Table IV). The T_1 for LP-X is 2.23 s, within experimental uncertainty of the 2.53 s observed for the phosphatidylcholine–cholesterol vesicle system. Vesicles containing no cholesterol had a T_1 of 2.33

Table IV: Spin-Lattice Relaxation Times (T_1) and Line Widths ($\nu_{1/2}$) of Phosphatidylcholine Phosphorus Resonances in LP-X and Selected Related Systems[a]

system	$\nu_{1/2}$ (Hz)	T_1 (s)
LP-X_1	20	2.23 ± 0.15
EYPC-UC	17	2.53 ± 0.19
EYPC	7-10	2.33 ± 0.12
LDL	5-7	2.19 ± 0.14
HDL	4-6	2.07 ± 0.15

[a] T_1 measurements were done with a pulse width of 23 μs (180°) and 11.5 μs (90°), using ⩾7 different delay times. Actual T_1 values were determined with an iterative fit of line intensities (256 or 512 scans) to the exponential $A + Be^{-\tau/T_1}$. Sweep width = 2000 Hz; indicated errors are the standard deviation between the calculated function and the experimental data. T_1 values are considered to be accurate to ±10%. Single-bilayer vesicles of egg yolk phosphatidylcholine (EYPC) with and without cholesterol ($x_c = 0.4$) were prepared by the method of Newman & Huang (1975).

s, virtually the same as that for LP-X. The T_1 value for these three vesicular systems was not very much above that observed for LDL (2.19 s) or HDL (2.07 s), particles shown by X-ray scattering studies to have micellar structures (Laggner & Müller, 1978). The interpretation of these similarities is not straightforward since the sensitivity of ^{31}P T_1 values to dynamic changes, particularly in anisotropic systems, is not well established. In the present study, line widths appear to be more sensitive to structural differences between the lipoproteins and model systems than are T_1 values. Line widths of 4–6 Hz for HDL and 5–7 Hz for LDL are significantly less than those of the larger vesicular systems.

Discussion

The 60- and 100-MHz spectra of human LDL (Steim et al., 1968; Leslie et al., 1969), the 100-MHz spectrum of rat LDL (Fidge & Calder, 1972), and the 220- and 100-MHz spectra of pig LDL (Finer et al., 1975) have been reported. While all of these proton spectra contain the easily observable cholesteryl–acyl CH_2 and CH_3 envelopes as well as the choline CH_3 resonance, the resolution of other less intense resonances (Table I) is significantly better in the present study. Nonetheless, it is apparent from inspection of the ^1H (Figure 1) and ^{13}C [Figure 1A of Brainard et al. (1980)] spectra that the carbon spectrum is significantly richer in detail and of greater potential utility in studying the motional properties of different molecular types in lipoproteins.

The temperature dependence of the porcine LDL_2 proton spectrum appears to be somewhat different from that of human LDL. At 30 °C and 220 MHz, the cholesteryl–acyl CH_2 and CH_3 lines have widths of 34 and 24 Hz in porcine LDL_2, while the corresponding line widths for human LDL are 42 and 27 Hz, respectively [Figure 2 of Finer et al. (1975)].[4] While the width of the cholesteryl–acyl CH_2 envelope in porcine LDL_2 decreases monotonically with increasing temperature, reaching a minimum at ~60 °C, in human LDL this minimum is reached at ~40 °C (Figure 2A). The shapes of the heating curves for the choline methyl and cholesteryl–acyl methyl line widths of porcine LDL_2 are comparable to those for human LDL, although direct comparison of absolute values is not possible due to differences in resonance frequency for the two experiments. In general, however, it would appear that porcine LDL_2 has a somewhat looser structure in which motion of the lipids is less constrained than it is in the human counterpart.

The results of the Tempo permeability experiments indicate widely differing permeabilities (fluidities) for LDL and LP-X. Although previous EPR studies (Patsch et al., 1977) indicated that Tempo is largely excluded from hydrophobic domains of LP-X, the location of these domains was unknown. The present NMR studies more precisely indicate that the penetration of this amphiphile into regions containing cholesteryl and acyl methyl and methylene groups is prevented (Figure 3B). In contrast, Tempo partitions extensively into these regions of LDL. The line broadening effects are particularly dramatic for the methylene and methyl envelopes of LDL, relative to both the choline methyl resonances of LDL and all the major proton resonances of LP-X. This observation suggests that Tempo penetrates preferentially into the hydrophobic core of LDL and remains there a relatively long time. Previous EPR experiments indicated that the residence time had a lower limit of 1.1×10^{-10} s. The present NMR experiments increase this limit to 4.5×10^{-9} s. In addition, the broadening effect of Tempo on the $(CH_3)_3N^+$ resonance is greater for LDL than for LP-X. This observation suggests that a fraction of the choline methyl groups in LP-X are shielded from the paramagnetic relaxing effect of Tempo. This shielding may be due to (a) protection afforded by the apoprotein or cholesterol or (b) the existence of a fraction of choline methyl groups in a location spatially different from that of the affected fraction. We believe that the first possibility is rather remote since LP-X contains such a small fraction of protein (~5%) and since cholesterol has been shown not to interact strongly with the choline methyl groups (Stockton et al., 1974). The second possibility is consistent with a vesicle structure for LP-X in which there is an external population of choline methyl groups accessible by Tempo and thereby susceptible to its line broadening effects and an internal population inaccessible and thereby resistant to these effects. Although Sillerud & Barnett (1977) have shown that Tempo can easily penetrate a fluid bilayer of pure egg phosphatidylcholine as evidenced by equivalent broadening of the inside and outside choline methyl protons, our results indicate that Tempo does not readily penetrate LP-X. Apparently, the presence of cholesterol within the LP-X bilayer renders it impermeable to Tempo, thereby preventing the probe from reaching the inside monolayer.

Tempo does not induce the same magnitude of line broadening for ^{13}C resonances as it does for ^1H resonances. For example, at 12 mM, Tempo broadens the LDL choline $N^+(CH_3)_3$ proton resonance from 9 to 40 Hz, whereas the corresponding ^{13}C resonance is not detectably changed.[3] This difference can be understood in terms of the equation which describes the nuclear resonance relaxation time in the presence of a paramagnetic species (Solomon, 1955):

$$\frac{1}{T_{2m}} = \frac{1}{15r^6}S(S+1)\gamma_I^2 g^2\beta^2\left[4\tau_c + \frac{\tau_c}{1+(\omega_I-\omega_S)^2\tau^2} + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{6\tau_c}{1+\omega_S^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_I+\omega_S)^2\tau_c^2}\right] \quad (1)$$

where S = the electronic spin quantum number, γ_I = the nuclear gyromagnetic ratio, g = electronic g factor, β = Bohr magneton, τ_c = the effective correlation time of the vector between the relaxing nucleus and the nitroxyl group, r = distance between the relaxing nucleus and the nitroxyl group, ω_I = the nuclear Larmor precession frequency, and ω_S = the

[4] This difference in line widths is not attributable to particle size differences. The diameter of porcine LDL_2 and human LDL is 215 and 218 Å, respectively, as determined by electron microscopy. By quasi-elastic light scattering, these diameters are 258 and 229 Å (Jackson et al., 1976). These diameters would suggest that porcine LDL_2 would have the broader lines, which is just opposite the experimental observation.

electronic Larmor precession frequency. Hence, the ratio of line widths for a proton and carbon nucleus in the presence of Tempo is

$$\nu_H/\nu_C = \gamma_H{}^2 r_{O\text{-}H}{}^{-6}/(\gamma_C{}^2 r_{O\text{-}H}{}^{-6}) \quad (2)$$

Examination of CPK space-filling models indicates that the minimum possible center-to-center distance between a nitroxyl oxygen and a hydrocarbon proton (carbon atom) is ~2 Å (3 Å). Since the proton and carbon gyromagnetic ratios are 26 753 and 6728 rad s^{-1} G^{-1}, respectively, the above ratio becomes

$$\nu_H/\nu_C = 180/1 \quad (3)$$

Hence, a given concentration of spin-label will be ~180 times more effective in broadening the resonance line of a proton than of a carbon nucleus in the same molecule.

The data presented in Table III and Figure 4 provide strong evidence supporting the view that LP-X is a bilayer vesicle whose Pr^{3+} titration behavior closely resembles that of an extensively studied and well-understood model system, egg phosphatidylcholine vesicles. The extreme stability and impermeability of LP-X and a similar model system consisting of phosphatidylcholine–cholesterol vesicles to which apoC and albumin have been bound is illustrated in Figure 4. The sidedness of the native and model lipoproteins persists even after extensive incubation at elevated temperatures. Hauser et al. (1977) have observed that there is no leakage of externally added polyvalent cation (Gd^{3+}) or anion ($FeCN_6^{3-}$) to the internal aqueous space under normal conditions and that even a monovalent cation (Na^+) was incorporated only when LP-X was sonicated.

The excellent resolution between resonances attributable to the internally and externally oriented phosphate groups allows accurate integration of the areas under these curves and subsequent calculations of the ratio of phospholipids on the internal and external surfaces of the vesicle (Table III). EYPC vesicles show phospholipid distributions consistent with the surface area ratios calculated from reported physical dimensions of the vesicles. In contrast, the phospholipids in LP-X do not appear to distribute in the ratio predicted by the surface area calculations (Table III). Several investigators have noted that inclusion of cholesterol in sonicated, unsaturated phospholipid vesicles is associated with an increase in the ratio of accessible to inaccessible phosphate groups, e.g., 2.2 → 2.9 (Huang et al., 1975) and ~2 → 2.7 (De Kruijff et al., 1976). These authors have interpreted their results as indicating that cholesterol has a preference for the inner monolayer, a preference which has been attributed to vesicle curvature and lipid packing effects. The distribution of phosphate groups in LP-X suggests that cholesterol has a slight preference for the outer monolayer. Phosphorus spin–lattice relaxation times were observed to be similar in LP-X, LDL, and HDL and in EYPC vesicles with and without cholesterol. However, the phosphorus spin–lattice relaxation time does not appear to be very sensitive to the presence of cholesterol in the phospholipid bilayer. Phosphatidylcholine vesicles containing 40 mol % cholesterol gave a T_1 value which was within experimental error of vesicles containing no cholesterol (Table IV). Although our reported T_1 values are slightly longer than those reported by Yeagle et al. (1975), both that study and the present one indicate that incorporation of cholesterol into the vesicles changes the T_1 only slightly. Apparently, those motions which determine the phosphorus spin–lattice relaxation time are very similar for all the systems studied. In contrast, Cullis (1976) has pointed out that the increase in line width with increasing cholesterol content may be related solely to an increase in vesicle size and may not necessarily reflect changes in the local motions of the phospholipid phosphate moiety resulting from the presence of cholesterol. There is considerable evidence that cholesterol does not significantly affect the motions of the phosphate moieties in phospholipid bilayers (Brown & Seelig, 1978; Yeagle et al., 1975). Consequently, we believe that the differences in line widths for the different particles studied probably reflect variations in their rotational diffusion constants.

Acknowledgments

The authors are indebted to Douglas Armstrong, David Scupham, and Susan Pettibone for technical assistance, to Dr. Judith Harmony for numerous helpful discussions and extensive use of laboratory facilities, and to Kaye Shewmaker and Debbie Mason for help in preparing the manuscript (TTGA).

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A Theory of the Effects of Head-Group Structure and Chain Unsaturation on the Chain Melting Transition of Phospholipid Dispersions†

Charles B. Berde,‡ Hans C. Andersen,* and Bruce S. Hudson§

ABSTRACT: We have developed statistical mechanical descriptions of the effects of head-group structure and acyl chain unsaturation on the chain melting phase transition of aqueous dispersions of bilayers containing glycerophosphocholines and glycerophosphoethanolamines. The theoretical framework is an extension of the model of Jacobs et al. [Jacobs, R. E., Hudson, B. S., & Andersen, H. C. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3993]. There are several systematic trends in the experimental transition data for various types of phospholipids. Assumptions about the physical origins of these trends were incorporated into statistical mechanical models, which were used to calculate transition temperatures and enthalpies. The extent to which the calculated results of a model reproduce the experimental trends is taken as a measure of the validity of the assumptions on which the model is based. We found that the gross differences among the transition temperatures of phospholipids with two saturated chains, two trans-unsaturated chains, two cis-unsaturated chains, and one cis-unsaturated and one saturated chain can all be explained in terms of the effect of the double bonds on molecular shape and the subsequent effect of shape on the ability of molecules to pack together into a low-energy state at high density. The dependence of transition temperature on the location of the double bond in cis-unsaturated molecules can be understood on the same basis. The differences between the transition temperatures of glycerophosphocholines and glycerophosphoethanolamines with the same hydrocarbon chains can be explained in terms of a larger intermolecular attraction (or smaller repulsion) for the latter than for the former. These differences depend on the presence or absence of unsaturation in the hydrocarbon chains in a way that is consistent with the postulate that hydrogen bonding between glycerophosphoethanolamines is responsible for the differences.

The structure and properties of cell membranes are affected by the molecular structure of the lipids contained within it. For phospholipids, the nature of the head group, the length of the acyl chains, the degree of unsaturation of the chains, and, in the case of acidic phospholipids, the extent of deprotonation and divalent cation binding can all have an effect on the membrane's properties. One of the ways to study the relationship between lipid structure and membrane properties is to investigate the chain melting phase transition that takes place in synthetic phospholipid dispersions, which serve as simple models for the lipid domains of biological membranes (Ladbrooke & Chapman, 1969; Steim et al., 1969; Engleman, 1970; Reinert & Steim, 1970; Melchior et al., 1970; Ashe & Steim, 1971; Hubbell & McConnell, 1971; Lippert & Peticolas, 1971; Hinz & Sturtevant, 1972; Schechter et al., 1972; Nagle, 1973a,b; Marčelja, 1974; Marsh, 1974; Ranck et al., 1974; Träuble & Eibl, 1974; Overath et al., 1975; Jacobs et al., 1975; McCammon & Deutch, 1975; Linden & Fox, 1975; Morrisett et al., 1975; Cronan & Gelmann, 1975; Sklar et al., 1975, 1976, 1977; Mabrey & Sturtevant, 1976; Melchior & Steim, 1976; Thilo & Overath, 1976; Tecoma et al., 1977).

Several workers have devised statistical mechanical models to describe the chain melting phase transition in bilayers (Nagle, 1973a, 1975, 1976; Marčelja, 1974; Jacobs et al., 1975, 1977; Scott, 1975, 1977; McCammon & Deutch, 1975; Jackson, 1976). These studies confirm that the disordering of the acyl chains in the interior of the bilayer can lead to a

† From the Department of Chemistry, Stanford University, Stanford, California 94305. *Received November 16, 1979; revised manuscript received May 5, 1980.* This work was supported by a grant from the National Institutes of Health (No. GM 23085). H.C.A. is a John Simon Guggenheim Memorial Fellow, 1976–1977.

‡ Present address: Children's Hospital Medical Center, Boston, MA 02115. Results are taken from a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree in Biophysics, Stanford University. Supported by National Institutes of Health Grant GM 01822 as a participant in the Medical Scientist Training Program.

§ National Institutes of Health Research Career Development Awardee (No. GM 00284). Present address: Department of Chemistry and Institute of Molecular Biology, University of Oregon, Eugene, OR 97403.

0006-2960/80/0419-4279$01.00/0