Acta Cient. Venezolana 34: 209-215, 1983

THERMAL EFFECTS IN HUMAN PLASMA HIGH DENSITY LIPOPROTEINS $(HDL)_3$: A ^{13}C-FT-NMR STUDY

V. León, T. Kirchhausen,* E. M. Avila and L. Mateu
Laboratorio de Resonancia Magnética. Centro de Física,
Laboratorio de Estructura Molecular, Centro de Biofísica
y Bioquímica, Laboratorio de Lipoproteínas.
Centro de Biofísica y Bioquímica.
Instituto Venezolano de Investigaciones Científicas (IVIC).
Apartado 1827, Caracas 1010A, Venezuela.

ABSTRACT

^{13}C-FT-NMR spectra of human plasma high density lipoproteins (HDL_3) have been recorded between 4° and 38° C. Changes in chemical shifts, linewidths and integrals at low temperatures of the cholesteryl ester resonances were completely reversible and are consistent with a reordering of neutral lipid molecules within the lipoprotein. High temperature (38° C) spectrum of thermally denatured HDL_3 does not show changes in the integrals of the corresponding resonances in native HDL_3 spectrum. This suggest that in the disrupted particle most of the molecules display a similar degree of freedom than in the intact HDL_3.

EFECTOS TERMICOS EN LIPOPROTEINAS DE ALTA DENSIDAD DE PLASMA HUMANO (HDL_3): ESTUDIO CON RESONANCIA MAGNETICA NUCLEAR DE ^{13}C

RESUMEN

Varios espectros de Resonancia Magnética Nuclear de ^{13}C fueron registrados de lipoproteínas de alta densidad de plasma humano (HDL_3) entre 4° C y 38° C. A bajas temperaturas, se observaron cambios importantes en los corrimientos químicos, los anchos de línea y las integrales de las resonancias correspondientes a los ésteres de colesterol; dichos cambios fueron completamente reversibles y son consistentes con un reordenamiento de los lípidos neutros en el interior de las lipoproteínas. A 38° C las integrales de las resonancias correspondientes a espectros de HDL_3 desnaturalizada térmicamente, resultaron muy similares a las de espectros de HDL_3 nativa registrados a igual temperatura. Estos resultados sugieren que muchos de los componentes moleculares constituyentes de la HDL_3, tienen igual grado de libertad en la lipoproteína intacta y desnaturalizada.

* Dirección actual: Dept. of Biochemistry and Molecular Biology,
Harvard University
7 Divinity Avenue, Cambridge. Mass. 02138, U.S.A.

Recibido: 9/11/82
Aceptado: 22/2/83

INTRODUCTION

Electron microscopy[1] and small-angle x-ray scattering[2,3,4,5] have indicated that human plasma high density lipoprotein (HDL_3) is a quasi-spherical particle of about 100 Å diameter consisting of an outer shell of phospholipid headgroups and proteins 10-15 Å thick, which surrounds an inner core of apolar lipids of about 85 Å diameter. There is no complete agreement about the detailed organization of neutral lipid components within these particles. This is an important aspect since it has been shown by several groups[6,7,8] that in human plasma low density lipoproteins (LDL) cholesteryl esters undergo a reversible thermal transition in the body temperature range. On the contrary, Tall *et al.*,[9] using a variety of physical techniques as differential scanning calorimetry and small-angle x-ray scattering and Avila *et al.*,[10] using ^{13}C-FT-NMR could not detect a similar thermal transition in HDL.

In this paper, we present a ^{13}C-FT-NMR study of HDL_3 over a temperature range of 4° to 38° C. Our data are consistent with a reordering of the cholesteryl ester molecules within the HDL_3 particle at low temperatures.

METHODS

Lipoprotein preparation

High density lipoproteins (HDL_3) were isolated in the density range of 1.120-1.210 g/ml from pooled freshly collected sera of four healthy, fasting adult male donors by ultracentrifugal flotation in KBr in presence of 5×10^{-4} M EDTA, followed by one washing at density of 1.250 g/ml to eliminate albumin contamination.[11] HDL_3 was collected and dialyzed over forty eight hours against 5mM TRIS-HCL buffer, pH 7.2, containing 150 mM NaCl and 0.5 mM Na_2 EDTA. Purity of this lipoprotein fraction was determined by 0.5 % agarose electrophoresis in a buffer containing 0.2 M glycine and 25 mM TRIS-HCL, pH 8,6. Chemical composition was estimated by methods previously reported.[12] The protein content was 49.5 %, the relative lipid molar distribution was: phospholipids 46 %, free cholesterol 14 %, cholesteryl esters 30 % and triglycerides 10 %; molecular weights adopted as in.[5]

The purified HDL_3 was subsequently concentrated to about 100 mg HDL_3/ml by dialysis against dry Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala): 4 ml of this sample were used for NMR study. All steps were performed at 4° C.

^{13}C-FT-NMR measurements

NMR spectra were obtained at 25.16 MHz using a spectrometer consisting of: a Varian XL-100 magnet, a T1010-A pulse unit, 293-A programable pulser and a 1180 data system from Nicolet Instrument Corporation (NIC).

A NIC TT1025 interface unit was used for quadrature phase detection. Spectra were obtained using 2500 spectral width and 8192 time domain points. Fourier Transform was performed on twice this number of domain points. A digital broadening of 2Hz was used for the spectra shown in Figure 2

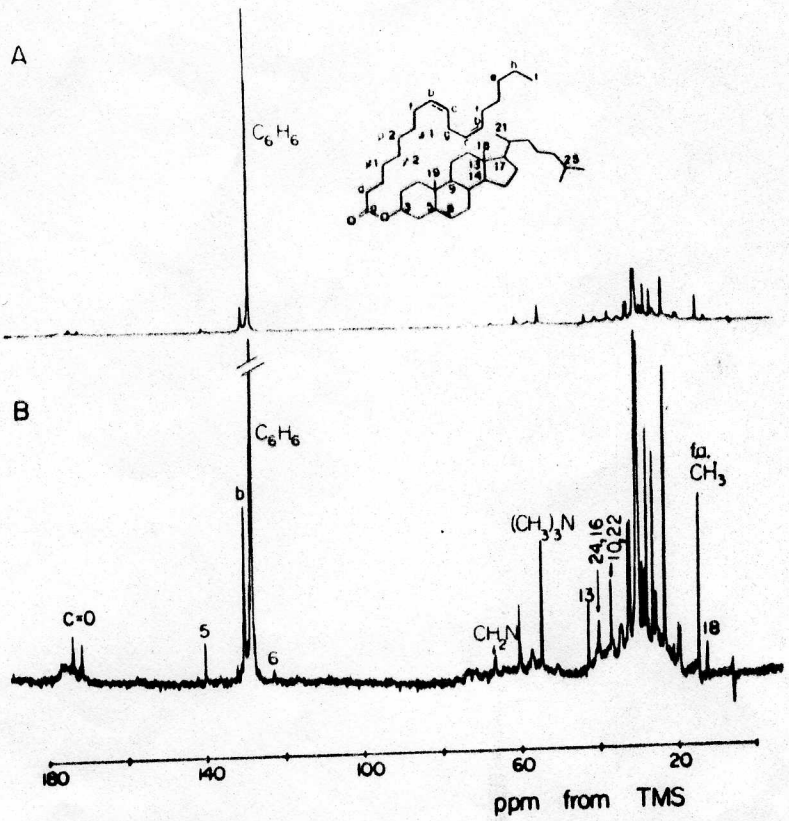

Figure 1A. ^{13}C-FT-NMR spectrum of HDL_1 recorded at 38° C. 26K accumulations, 1.7 sec recycle time. A cholesteryl ester molecule with the notation used in the assignments is included.

Figure 1B. 7 fold vertical expansion of the spectrum shown in A.

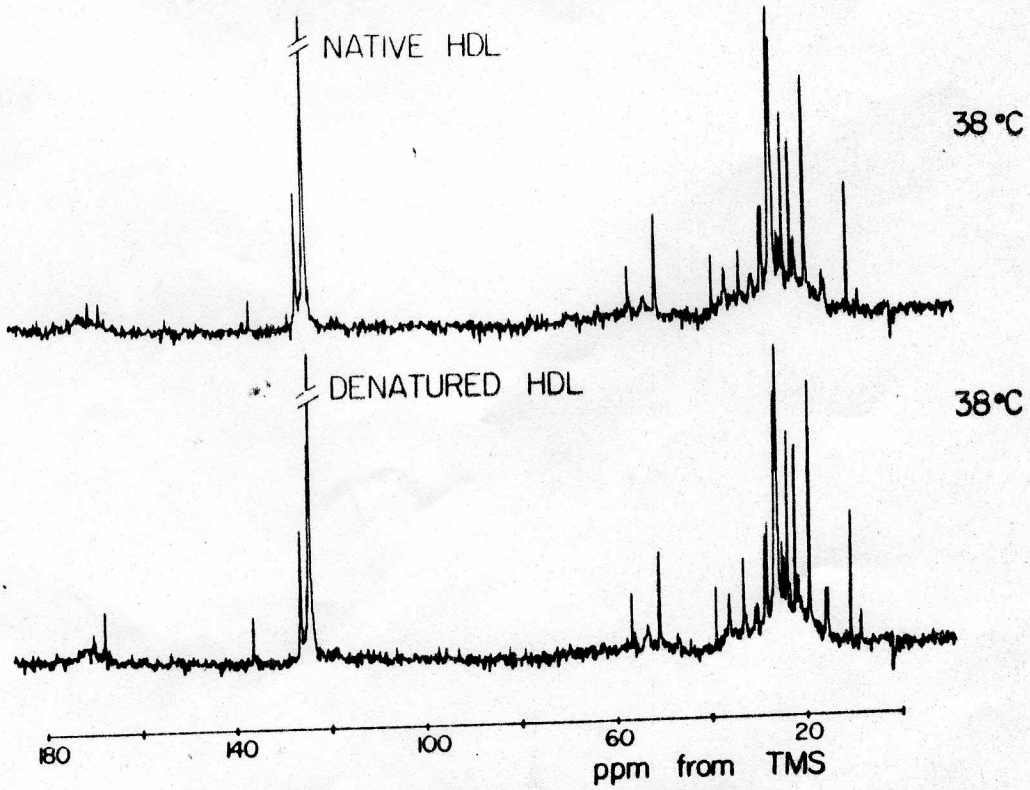

Figure 3. ^{13}C-FT-NMR spectra of native and thermally denatured HDL, recorded at 38° C. 6K accumulations, 1.7 sec recycle time. Spectra were normalized for plotting using the benzene integral.

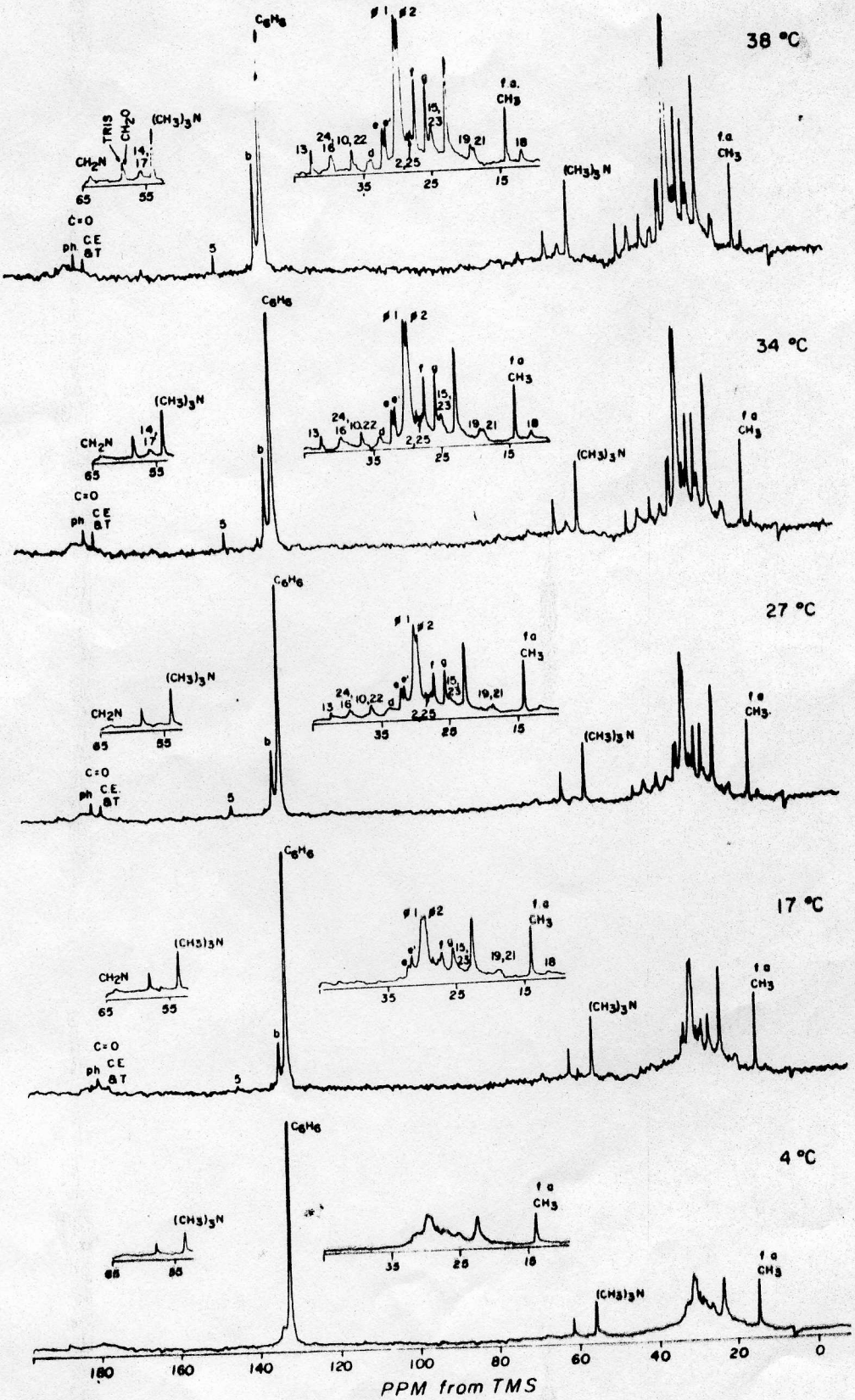

Figure 2. ^{13}C-FT-NMR spectra of HDL$_3$ recorded at 25.16 MHz, 1.7 sec recycle time. The number of accumulated transients was 6, 6, 4, 4, and 6K respectively for the 38, 34, 27, 17 and 4° C spectra. The spectra were normalized for plotting using the benzene integral. Inserts are 2/3 reduction, baseline corrected plots of the 67.79 to 52.15 PPM (left) and 45.07 to 9.19 PPM regions of the corresponding spectrum.

(analyzed in Table 1), and 0.25 Hz for the spectra shown in Figures 1 and 3. The H irradiation for proton decoupling was done using a random-noise modulation band width of about 2000 Hz, centered in the proton spectrum.

The 12 mm sample tube used included a 5 mm coaxial tube with deuterated acetone for locking, and benzene as internal reference and temperature control. Temperature of the sample was measured with a thermistor thermometer before and after each run. Temperature was stable within 1° C as was evident from the stability of the absolute frequency of the benzene carbon signal.[13]

Before each run four pre-equilibration pulses, at 1.7 sec recycle time, were used to reduce the long T_1 signals coming from benzene and deuterated acetone. PPM from TMS were measured assigning 14.1 PPM to the fatty acyl methyl carbon.

Data analysis

Figure 1A shows a spectrum recorded at 38° C in which relative intensity of benzene and HDL_3, resonances are compared. The resonance of benzene carbon, centered at 127.64 PPM, is about fifteen times more intense than the signal coming from olefinic carbons of acyl chains, centered at 128.35 PPM. Therefore, it was assumed that changes in olefinic resonance occurring with temperature, do not affect the integral of benzene carbon, used as internal reference. Chemical shifts and line widths were calculated using a line fitting program. Data points used for this purpose were only those in the region where any other contribution, different from the assigned resonance, could be discarded. Before doing any analysis spectrum was corrected from any deviation of baseline; this was done using a baseline straightening routine. Estimated errors were 0.02 PPM (0.5Hz) in chemical shifts and 10 % for linewidths.

Figures reported in Table I under | are partial relative normalized integrals of the corresponding signals. Procedure to calculate the corresponding | value was as follows: the first spectrum at 38° C (26K accumulations) was used to choose, for each resonance, and arbitrary and small region of the signal that was reasonably free from any other contributions (under υ_1/υ_2 symbol in Table I). Such region for benzene was integrated and normalized to 100 and the other integrals of the first spectrum, over the corresponding region for each resonance (υ_1/υ_2 in Table I) were measured relatively to this value. For any other spectrum, a line fitting for each resonance) was performed. Then, new υ_1/υ_2 regions were calculated scaling the corresponding regions of the first spectrum (26K accumulations at 38° C) proportionally to the new linewidths. The new corresponding region for benzene was normalized to 100 and all integrals over the new corresponding regions were calculated relative to this value. Using this procedure, we integrated the same portion of the line for all spectra. Estimated error for | was between 15 and 20 %.

RESULTS

Spectra of HDL_3 for different temperatures between 4 and 38° C are shown in Figure 2. Spectra were normalized for plotting using the benzene integral. Inserts for each spectrum were 2/3 reduction baseline corrected plots of two important regions of the spectrum. Assignments were done following previous ^{13}C-NMR data.[10, 14, 15, 18] Figure 1A shows a cholesteryl ester molecule indicating the notation used in the assignments. Table I, summarizes chemical shifts, linewidths and partial relative normalized integrals (see methods) for most resonances in the spectra. One additional spectrum at 38° C with better signal to noise ratio (spectrum B in Figure 1) is also analyzed in Table I.

There is a significant gradual shift of the resonance position for few resonances coming from the cholesterol ring, detectable up to 17° C: carbons 5, 13 and 18. This gradual shift is also present for the carbonyl resonance assigned to the cholesteryl esters and triglycerides, and for the *b* carbon, that has an important contribution of cholesteryl ester fatty acids. It is also clear that there is a decrease of the corresponding partial relative normalized integrals, that is, a decrease in the number of observable carbon nuclei, for these resonances. All other assigned resonances coming from the cholesterol ring became undetectable at 17° C. This is also true for the *d* resonance (see Figure 2) coming from the first carbon in the fatty acid linked to the cholesterol ring. This strong dependence with temperature contrasts with the weaker dependence of resonances for which contributions from carbons in triglycerides and fatty acids of phospholipids are important (ϕ_1, ϕ_2, e', e, g, f).

The $(CH_3)_3N$ resonance shows a very strong gradual dependence in resonance position with temperature with only a moderate decrease in partial relative normalized integral (35 % at 4° C). This behavior is similar for the CH_2N resonance. The fatty acid methyl carbon resonance shows a slight dependence in linewidth but with a constant relative partial normalized integral. All changes mentioned above were completely reversible with temperature. A spectrum recorded at 38° C after cooling at 4° C was identical to the initial spectrum recorded at 38° C.

Figure 3 shows ^{13}C-FT-NMR spectra for native and thermally denatured HDL_3, recorded at 38° C. Thermal denaturation was obtained by heating the lipoprotein sample for 10 min at 95° C.[9, 16] The denatured HDL_3 spectrum shows no change, within the estimated error in partial relative normalized integrals (|), for any of the corresponding resonances in the native HDL_3 spectrum. It is also evident from Table I, that there are slightly lower linewidth values for the corresponding denatured HDL_3 resonances. A further lipid segregation was attempted by cooling the denatured sample at minus 40° C for 20 minutes.[9, 16] However this treatment did not introduce new changes in the spectrum recorded subsequently at 38° C.

DISCUSSION

The results and conclusions of the present work can be considered to be valid for the chosen pulse delay (1.7 sec.) although for several carbons in the spectrum $4T_1$ is probably higher than 1.7.[15] This is because all the analysis have been made internally, based on relative quantities. Moreover, experiments performed at 38° C using pulse delay of 2.1 sec and 3 sec did not introduce important changes in the spectrum (not

shown). We have also found that the fatty acid terminal methyl is a good selection for internal normalization, even at , since the corresponding values are constants for all temperatures.

Resonances coming from the cholesterol ring carbons were found to be significantly temperature-dependent, most of them are non-detectable at 17° C. In addition, on lowering temperature, partial relative normalized integrals for carbons 5, 13 and 18 show a decreasing value concomitantly with an important gradual shift in resonance position. This can be interpreted as a reordering in the environment of cholesteryl esters which is characterized by a higher motional restriction of these molecules. It is evident that this effect is not an overall restriction with temperature for all the molecular components in the lipoprotein, from the weak dependence observable for other carbons (ϕ, ϕ_2, e', e, g, f) for which the contributions coming from triglycerides and phospholipids fatty acids are important. It has to be noted that with the use of partial relative normalized integrals, it is possible to follow the same proportion of carbons nuclei in spite of variations in linewidth, so that, any lower value of | (15-20 % error) has to be interpreted as a change in dynamics of the corresponding molecules, for instance, a shorter T_2 value due to a more «packed» arrangement.

The comparison between native and thermally denatured HDL$_3$ shows no changes, within the estimated error, in values for any of the corresponding resonances (see Table I). In agreement with Hamilton and Cordes,[18] our results suggest that there is no important pool of molecules in the native HDL$_3$ much more restricted in their motions than in the disrupted particle. However, there is evidence[9] that even after heating HDL$_3$ to 97° C and recooling, its molecular components could

TABLE I

Assignment		T = 38 °C	T = 38 °C	T = 34 °C	T = 27 °C	T = 17 °C	Denaturalized T = 4 °C	Denaturalized T = 38 °C	Comments
Benzene* (reference)	U_0	127.64	127.66	127.63	127.62	127.61	127.59	127.64	*reference for I
	$\Delta U_{1/2}$	38	40	36	34	36	36	36	
	I	100	100	100	100	100	100	100	
	U_1/U_2	229/-7.48	262/-7.76	188/-7.28	244/-6.71	250/-6.78	189/-6.65	241/-6.75	
C = O Phospholipids	U_0	173.56	173.56	173.56	173.52	173.50		173.61	
	$\Delta U_{1/2}$	7.9	6.2	4.9	7.6	8.4	ND	6.1	
	I	4.8	3.8	2.8	4.2	2.6		4.8	
	U_1/U_2	5.30/-6.29	4.10/-6.27	3.47/-3.85	5.34/-6.26	5.53/-6.68		4.19/-4.96	
C = O Cholesterol ester & triglycerides	U_0	171.29	171.30	171.26	171.20	171.12		171.20	
	$\Delta U_{1/2}$	6.0	5.4	3.9	6.8	7.7	N.D	4.7	
	I	3.3	2.8	2.6	3.7	1.3		4.4	
	U_1/U_2	4.34/-6.04	3.63/-6.13	2.88/-3.83	5.05/-6.54	5.61/-7.21		3.04/-4.90	
5	U_0	139.80	139.79	139.74	139.66	139.65		139.83	
	$\Delta U_{1/2}$	4.8	5.4	5.5	7.2	7.2	N.D	4.8	
	I	2.7	2.9	3.4	3.3	1.2		4.4	
	U_1/U_2	2.84/-3.87	3.00/-4.30	3.69/-4.85	6.57/-8.69	4.12/-5.65		2.58/-4.13	
	U_0	129.74	129.75	129.72	129.64	129.61	*	129.73	* difficult to separate the other contributions
	$\Delta U_{1/2}$	8.1	9.9	11	11	13		8	
	I	2.2	2.6	2.6	1.9	1.6		2.1	
	U_1/U_2	5.64/-6.57	6.61/-8.04	7.45/-8.42	8.84/-9.22	8.98/-10.55		4.69/-6.30	
6	U_0	122.32	122.34	**	N.D	N.D	N.D	122.26	**Artifact present
	$\Delta U_{1/2}$	14*	12*					19*	* low signal to noise ratio
	I	3.2	2.5					5	
	U_1/U_2	8.70/-10.83	7.72/-9.37					11.47-14.16	
3	U_0	73.04						73.17	
	$\Delta U_{1/2}$	2.5						1.3	
	I	2*	*	*	N.D	N.D	N.D	3	* very low signal to noise ratio
	U_1/U_2	2.002/-2.392						9.97-12.00	
CH_2N	U_0	66.24	66.27	66.20	66.07	66.01	65.98	66.32	
	$\Delta U_{1/2}$	1.3	1.5	1.4	2.7	1.4	2.0	1.5	
	I	4.2	4.5	3.9	3.3	3.3	2.6	1.4*	* low signal to noise ratio
	U_1/U_2	10.36/-15.27	9.14/-13.44	11.35/-16.72	21.67/-32.19	11.34/-16.13	16.56/-23.73	9.82/-15.20	
CH_2O	U_0	59.83	59.77	59.73	59.69				* too difficult to separate the contribution of the sharp resonance coming from the TRIS buffer
	$\Delta U_{1/2}$	1.5	1.1	1.1	1.4	*	*	*	
	I	3.9	3.0	3.2	2.6*				
	U_1/U_2	1.56/-12.38	1.16/-9.21	8.71/-8.89	9.27/-11.29				
14,17	U_0	56.73	56.71	56.80	56.72			56.76	* There are apparently three small peaks.
	$\Delta U_{1/2}$	2.5	2.1	2.4	2.9	*	ND	1.8	
	I	8.7	8.3	6.9	3.7			0.9	
	U_1/U_2	15.28/-15.85	12.87/-13.38	14.92/-15.60	18.12/-18.33			11.57/-11.63	
$(CH_3)_3N$	U_0	54.26	54.26	54.19	54.12	53.96	53.80	54.28	
	$\Delta U_{1/2}$	7.4	8.0	6.7	7.1	7.5	9.3	8.0	
	I	1.8	2.0	1.6	1.3	1.5	1.2	1.8	
	U_1/U_2	6.63/-10.46	7.43/-10.88	6.01/-9.24	6.73/-9.46	6.79/-10.91	8.24/-13.12	6.90/-11.41	
9	U_0	50.24						50.29	* too low signal to noise ratio
	$\Delta U_{1/2}$	1.7	*	*	N.D	N.D	ND	1.5	
	I	2.9						4.3	
	U_1/U_2	17.54/-19.69						15.81/-17.76	
13	U_0	42.42	42.41	42.38	42.31	42.19		42.43	
	$\Delta U_{1/2}$	3.8	3.9	3.5	4.0	4.0	N.D	3.3	
	I	3.9	4.4	2.6	1.5	1.2		3.4	
	U_1/U_2	3.85/-5.30	3.99/-5.16	3.05/-4.88	3.59/-5.28	4.10/-5.70		3.49/-4.44	
24,16	U_0	39.71	39.69	39.64	39.70			39.71	
	$\Delta U_{1/2}$	1.2	1.2	1.1	1.6	N.D	N.D	1.1	
	I	9.2	8.9	7.0	6.4			8.8	
	U_1/U_2	9.70/-14.72	9.67/-14.75	9.13/-13.18	12.51/-19.64			9.02/-4.41	
10,22	U_0	36.60	36.61	36.57	36.55			36.62	
	$\Delta U_{1/2}$	4.9	5.0	4.3	1.0	ND	ND	4.6	
	I	5.1	4.3	3.3	5.0			5.4	
	U_1/U_2	4.93/-2.40	4.76/-2.56	4.48/-2.00	9.52/-5.12			4.54/-2.17	
d	U_0	34.00	34.02	34.04					* too low signal to noise ratio ** there is some structure present.
	$\Delta U_{1/2}$	2.0	1.7	1.3	*	*	ND	**	
	I	1.1	7.5	6.7					
	U_1/U_2	1.89/-1.706	1.558/-1.372	1.254/-1.127					
e	U_0	32.07	32.07	32.07	32.07	32.12		32.09	
	$\Delta U_{1/2}$	6.7	6.8	6.4	10.0	9.6		7.2	
	I	1.3	1.2	1.2	1.2	7.4		1.5	
	U_1/U_2	5.99/-4.99	6.06/-4.93	5.92/-5.06	9.13/-6.74	8.34/-6.91		6.70/-4.90	
e'	U_0	31.62	31.63	31.60	31.59	31.57		31.64	
	$\Delta U_{1/2}$	5.4	5.6	5.6	8.0	8.6		5.7	
	I	9.7	9.1	1.0	1.0	9.4		1.1	
	U_1/U_2	3.87/-4.06	3.83/-4.11	4.39/-4.16	5.35/-6.25	6.35/-6.47		4.02/-4.53	

TABLE 1 (cont.)

Φ_1	υ_0	29.91	29.91	29.93	29.94	29.91		29.94	* includes part of Φ_2
	$\Delta\upsilon_{1/2}$	12	12	13	16	23		14	
	I	63	65	61	53	58*		66	
	υ_1/υ_2	1219/-428	1221/-427	1353/-417	1635/-562	2431/-804		1401/-491	
Φ_2	υ_0	29.48	29.49	29.48	29.50	29.55		29.50	* includes part of Φ_1
	$\Delta\upsilon_{1/2}$	14	15	15	15	20		12	
	I	58	65	56	36	42*		53	
	υ_1/υ_2	407/-996	436/-1090	399/-1065	422/-1104	602/-1474		363/-858	
2.25	υ_0	28.04	28.04	28.02				28.05	* too low signal to noise ratio
	$\Delta\upsilon_{1/2}$	90	13	14	*	ND	ND	88	
	I	86	12	11				90	
	υ_1/υ_2	300/-371	444/-533	456/-553				288/-383	
f	υ_0	27.27	27.27	27.24	27.24	27.22		27.28	
	$\Delta\upsilon_{1/2}$	65	70	69	86	15		64	
	I	21	23	24	14	17		22	
	υ_1/υ_2	416/-499	424/-552	597/-747	513/-647	907/-1107		446/-469	
g	υ_0	25.68	25.67	25.64	25.62	25.54		25.68	
	$\Delta\upsilon_{1/2}$	60	64	66	79	12		60	
	I	19	20	20	14	16		21	
	υ_1/υ_2	450/-405	473/-442	477/-439	616/-543	913/-796		437/-418	
15,23**	υ_0	24.99	24.98	24.96	25.02			25.02***	*some doubts of other contributions to the assignments **two peaks are apparent
	$\Delta\upsilon_{1/2}$	16	17	22	23	ND	ND	15	
	I	16	18	19	11			15	
	υ_1/υ_2	581/-701	632/-711	795/-975	832/-999			571/-650	
19	υ_0	19.33	19.31					19.34	* it is forming one peak together with 21
	$\Delta\upsilon_{1/2}$	86	98	*	*	*	ND	69	
	I	58	76					60	
	υ_1/υ_2	784/-315	913/-369					644/-272	
21	υ_0	18.88	18.85					18.86	* it is forming one peak together with 19
	$\Delta\upsilon_{1/2}$	70	85	*	*	*	ND	48	
	I	50	61					47	
	υ_1/υ_2	349/-750	434/-909					269/-525	
f.a * CH_3	υ_0	1411	1410	1411	1411	1411	1411	1410	* Reference for chemical shifts (141 PPM from TMS)
	$\Delta\upsilon_{1/2}$	42	47	45	45	50	64	42	
	I	13	14	15	12	14	12	13	
	υ_1/υ_2	820/-644	894/-693	875/-711	885/-702	981/-786	1250/-947	828/-636	
18	υ_0	1197	1197	1196	1191	1190		1196	
	$\Delta\upsilon_{1/2}$	70	76	11	88	13	ND	62	
	I	47	52	54	27	33		48	
	υ_1/υ_2	709/-756	775/-811	1149/-1171	868/-963	1308/-1438		656/-687	

* Assignments for the cholesteryl ester molecule are shown in Figure 1A, other assignments are self explanatory.

| Are the partial relative normalized integrals (see methods).

υ_1/υ_2 Is the region, in Hz, below and above of the resonance position chosen for integration (see methods).

υ_0 Are the chemical shifts in PPM from TMS.

$\Delta\upsilon_{1/2}$ Are the linewidhts on Hz. No substraction of the 2 Hz of digital broadening was made.

N.D. Is not defined.

retain organized structures in large spheres and disc like particles. This fact could explain the similarities between NMR spectra of native and denatured HDL_3 at 38° C. Nevertheless, there is a small overall improvement of the degree of freedom, reflected in smaller linewidths for most of the resonances in the spectrum of the disrupted HDL_3. So, the suggestion of a strong interaction between cholesteryl esters and the protein-phospholipid surface to explain the absence of a transition in native HDL_3[9] could be interpreted differently. One possibility would be that we are probably detecting a remanent microscopic behavior in the cholesteryl ester molecules smoothed out by the high content of triglycerides[20, 21] and probably by the small size of HDL_3.[22] So, the NMR technique allows to follow microscopic dynamical changes even without (or before) any macroscopic manifestness. Therefore, an interesting finding is that individual molecules in HDL_3 undergo temperature related transitions, but non-cooperative transitions.

Our results should not be considered in contradiction with those previously reported by other groups[10, 17] since the more significant changes described here occur at low temperature, out of the range previously reported. With respect to a pool of free cholesterol motionally restricted within the native HDL_3 particle, previously suggested,[10] we cannot discard that possibility because the corresponding changes in the values (of the denatured HDL_3 spectrum) would be of the order of 20 %, the size of our estimated error. In addition, we cannot discard a motionally restricted pool of free cholesterol in the denatured HDL_3 or even in the mixture of segregated lipid components without following a complete solubilization.

Finally the behaviour of phospholipid head groups with temperature reflected by the choline resonance, namely, a strong shift in resonance position and a small decrease of the values, is indicative of changes in the surface of HDL_3 particles with temperature, so that parallel with changes in the arrangement of the «lipid core» there are also changes in the surface structure of the lipoprotein.

AKNOWLEDGMENTS

This work was supported in part by a research project granted from CONICIT to L. M. We thanks Miss O. Arandia and L. Sevcik for secretarial work. Leonardo Mateu is an Associate of the Instituto Internacional de Estudios Avanzados (IDEA).

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