Physical studies of d < 1.006 g/ml lymph lipoproteins from rats fed palmitate-rich diets

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Abstract At body temperature the stable form of triglycerides rich in saturated fatty acids is crystalline. We examined the physical state of triglyceride-rich lymph lipoproteins from rats fed saturated fat, as a function of temperature. When chylomicrons and very low density lipoproteins were collected, isolated, and examined at 37°C, they were liquid as judged by differential scanning calorimetry, x-ray diffraction analysis, and proton nuclear magnetic resonance spectroscopy, and they appeared spherical by electron microscopy. At 23–26°C, triglyceride began to crystallize in the α form, which transformed to the stable β form at lower temperatures. On cooling from 23°C to 17°C, considerable crystallization occurred and the particle density was increased significantly. When lipoproteins were held at 0–7°C, about 75% of the triglyceride crystallized, distorting the lipoprotein shape. Reheating from 0°C to 37°C left 25% of the triglyceride unmelted. Heating to 58°C was necessary to melt all the crystallized triglyceride and to restore the spherical lipoprotein shape. After complete melting of cooled lipoproteins, the liquid state was maintained on recooling to 37°C, with formation of a metastable particle similar to the nascent lipoprotein. Isolation of lipoproteins containing highly saturated triglyceride at temperatures below 23–26°C results in partial crystallization, alters their physical properties, and may affect their metabolism.—**Bennett Clark, S., D. Atkinson, J. A. Hamilton, T. Forte, B. Russell, E. B. Feldman, and D. M. Small.** Physical studies of d < 1.006 g/ml lymph lipoproteins from rats fed palmitate-rich diets. *J. Lipid Res.* 1982. **23:** 28–41.

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Intact lipoproteins (LP), such as low density lipoproteins (LDL) from normal human plasma (1), show phase transitions during heating and cooling through temperatures encompassing body temperature. Studies from this laboratory established that these phase changes in LDL are due to phase changes in the cholesterol esters that comprise the LP core (1, 2). Cholesterol ester-rich LP from swine (3, 4), monkeys (5), cattle (6), and rabbits (7) also have been shown to undergo similar transitions. It seems probable that the physical properties of all lipid-rich LP are strongly influenced by the properties of their component lipids.

Lipoprotein isolations are typically performed at temperatures between 4–15°C, a temperature range that encompasses the crystallization temperatures of triglycerides (TG) rich in saturated fatty acids (8, 9). We chose to study dietary LP particles secreted in intestinal lymph because their triglyceride-fatty acid (TG-FA) composition can be modified readily by feeding diets of defined FA composition (10–13). Thus, TG-rich lymph LP, which are secreted during ingestion of diets rich in saturated fat, contain significant amounts of saturated fatty acids (14). The physical properties of chylomicrons and very low density lipoproteins (VLDL) formed under these conditions probably are influenced by the temperature at which they are isolated. Recently, Puppione et al. (15) described a novel TG-rich LP obtained from bovine plasma and lymph, which was characterized by flat appearance, asymmetric shape, and anomalous density after isolation at 4–16°C. It was suggested that this LP was an artifact formed by the crystallization of the saturated TG present within the LP. During the course of our dietary studies in rats (12–14), electron micrographs of chylomicrons and VLDL isolated at 0–4°C revealed similar irregular shapes and prompted the present in-depth study of the physical properties of these lipoproteins. Our experiments demonstrate that highly saturated LP-TG secreted by the intestine in native LP are present as metastable, undercooled liquids. Crystallization of TG and concommitant structural changes in the LP are produced on cooling below the TG nucleation temperature.

Abbreviations: LP, lipoprotein; TG, triglyceride; LDL, low density lipoprotein; VLDL, very low density lipoprotein; FA, fatty acid; TLC, thin-layer chromatography; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance.

METHODS

Lipoprotein collection and isolation

Male rats aged 6–7 weeks were fed a semi-synthetic fat-free diet, to which 15% (wt/wt) triglyceride containing 75% palmitate was added (13, 14), for 4 weeks. The major mesenteric lymphatic trunk was cannulated when the rats were in the fed state and food was removed. Twenty-four hr later they received, by gavage, 100 mg of tripalmitin suspended by sonication in 2 ml of Krebs-Ringer phosphate buffer containing 20 mM Na taurocholate (time 0). The animals were maintained in restraint cages in a constant temperature room held at 37°C and were allowed free access to 0.5 N NaCl containing 100 g/l dextrose. Eight hr after tripalmitin gavage, the high palmitate diet was again administered. Lymph was collected continuously, at 37°C, in two batches, i.e., 0 to 8 hr and 8 to 24 hr after gavage. The collection tubes contained EDTA, NaN_3, and DTNB to achieve final concentrations of 1 g/l, 1 g/l, and 4 mM, respectively.

Preparative ultracentrifugation of lymph chylomicrons and VLDL was begun on the day of lymph collection and was performed entirely at 37°C. Aliquots of lymph (9–10 ml) were overlayered with 2–3 ml of a d 1.006 g/ml solution, pH 7.4, containing NaCl (8.5 g/l), Na_2 EDTA (1.0 g/l), and NaN_3 (0.5 g/l), and the chylomicrons were floated in a swinging bucket rotor (SW 41) at 3×10^6 g·min. The top (chylomicron) fraction was harvested by tube slicing and the infranatant was spun at 10^8 g·min to float VLDL. Lipoprotein fractions containing NaN_3 were stored at 37°C and at no time were they allowed to cool below 27°C prior to physical examination. For x-ray diffraction experiments, chylomicrons were further concentrated by recentrifugation under the same conditions. Compositional analysis (see below) of aliquots taken immediately after isolation and at the end of the physical studies confirmed that LP degradation by LCAT and lipoprotein lipase were negligible.

Lipid analysis

Aliquots of the four chylomicron and four VLDL fractions were extracted with chloroform–methanol 2:1 (16) and the total lipid contents were determined gravimetrically, using a Cahn balance (Model 25). Neutral and polar lipid classes were separated by thin-layer chromatography (TLC) and were quantitated by charring and densitometry, essentially as previously described (17), except that tripalmitin replaced triolein as the TG standard used to calculate TG mass. Glyceride-glycerol was also estimated spectrophotometrically (18). TG fatty acids were determined by gas–liquid chromatography of the methyl esters (19) after TLC fractionation of the lipid classes and elution of TG.

Differential scanning calorimetry

Aliquots (70 μl) of each lymph chylomicron and VLDL sample, containing 1.3–4.1 mg lipoprotein TG in d 1.006 g/ml saline solution, were placed in weighed DSC pans prewarmed to 37°C. Reference pans contained 70 μl of the saline solution.

To compare the LP with their component lipids, lipid extracts (16) of one chylomicron and one VLDL sample were concentrated and transferred to weighed DSC pans. The solvent was removed under N_2 and the pans, containing 2.5 mg of chylomicron-TG and 6.2 mg of VLDL-TG, respectively, were lyophilized overnight. Reference pans were empty.

Lipoproteins and LP lipids were studied in a Perkin-Elmer DSC-2 differential scanning calorimeter at a sensitivity of 0.1–0.2 mcal/sec and heating and cooling rates of 5°C/min, except where otherwise noted. Preliminary experiments using non-denatured LP established that the enthalpy changes (ΔH) and peak temperatures (T_p) of the major melting transitions, and the onset (T_c) and peak (T_p) crystallization temperatures during cooling, were the same within experimental error when heating and cooling rates were between 1.25–10°C/min.

For each LP sample, the first scan was a heating run from 37°C to 60°C, that is, from the temperature at which the native LP was secreted to just above the melting point of tripalmitin. Several heating and cooling runs were then performed between 37°C and 60°C. Subsequently, the LP samples were cooled several times to temperatures below 27°C, with each cooling run followed by a heating run to 60°C. The LP was then held between 0°C and −3°C for various times and was again heated to 60°C. Finally, the LP was heated to 90°C to denature and disrupt the particles. Cooling and reheating was then repeated on the denatured LP as described above. Major transitions were defined by the parameters illustrated in **Fig. 1.**

X-ray diffraction

X-ray diffraction measurements were performed on chylomicrons from one 8–24 hr lymph collection (Rat 2). The LP was further concentrated approximately 4-fold by recentrifugation at 37°C and the sample, suspended in the d 1.006 g/ml saline solution, was sealed in a 1-mm I.D. Lindeman glass tube warmed to 37°C (Lindeman Corp., Indianapolis, IN). X-ray diffraction patterns were obtained over the range 1/60–1/2.5 $Å^{-1}$ using a Jarrell-Ash microfocus x-ray generator and slit-collimated Luzzati-Baro x-ray camera modified to in-

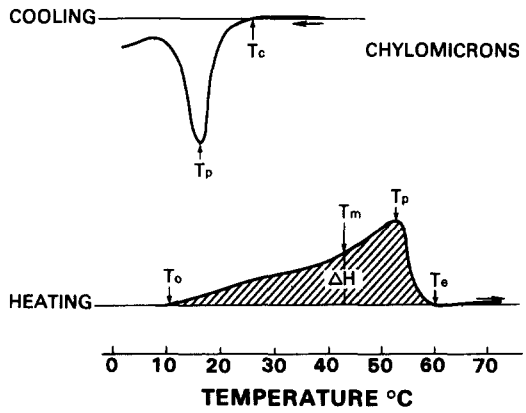

Fig. 1. Example of differential scanning calorimeter tracing defining major parameters obtained when chylomicrons, isolated at 37°C, are cooled and reheated. Cooling: T_c, crystallization onset temperature; T_p, peak crystallization temperature. Heating: T_o, onset of melting phase transition; T_m, melting transition half completed; T_p, peak of melting transition; T_e, end of melting transition; ΔH, integrated area under the curve, equivalent to the enthalpy change within the system.

clude a single mirror focussing system. Diffraction patterns were recorded for 10 min using a linear position sensitive counter (P.S.D. 1100, Tennelec, TN) coupled to a computer based analysis system (TN 1710, Tracor Northern, WI). Short spacings were estimated to ±0.1 Å and long spacings to ±3 Å, based on sample-detector distance and the accuracy with which the peak maxima could be located.

To compare the diffraction patterns of intact LP and LP lipids, an aliquot of VLDL from the same lymph collection was heat-denatured at 100°C for 10 min and the lipids were separated by high speed centrifugation in capillary tubes. The top, oil phase was transferred to a Lindeman tube and diffraction patterns obtained as above.

Proton magnetic resonance spectroscopy (NMR)

Fourier transform NMR spectra were obtained on a Bruker WP-200 Spectrometer System, operating at a field strength of 47 KGauss corresponding to proton observation at 200 MHz; the system was equipped with an Aspect 2000 Data System, a 5-mm proton probe, and quadrature detection.

NMR measurements were made on VLDL from the 8–24 hr lymph collection of Rat 2. The sample tube contained 21.1 mg of TG in 0.7 ml of the d 1.006 g/ml saline solution. D_2O (~10%) was added as an internal lock and shim signal and gated homonuclear decoupling was employed to suppress the H_2O peak. The pulse interval (4 sec) was sufficient to obtain equilibrium intensities for all peaks, based on spin lattice relaxation measurements in similar systems.[1] Subsequently, to obtain NMR spectra as a function of time, the sample was dialyzed against 10 ml of D_2O (Wilmad Co., 99.7% D) for 16 hr at room temperature, heated to 60°C, and concentrated 2-fold in a vacuum dessicator at 40°C.

Sample temperature in the NMR probe was regulated to ±1°C using a Bruker BVT-1000 Variable Temperature Unit; liquid nitrogen vapor was used to cool below 26°C. The sample was pre-equilibrated in a water bath at the planned probe temperature (±2°C) before each measurement, and was further equilibrated for 5 min in the probe before pulsing. After data acquisition for 10–15 min, the sample (containing no vortex plug) was ejected from the probe and its temperature was measured immediately (<1 min), using an Omega "Trendicator" (Model 410A) equipped with a thin thermocouple. The sample was then pre-equilibrated at the next temperature. This procedure avoided large temperature fluctuations (>2–3°C) between NMR experiments.

Proton peak assignments were made according to Finer, Flook, and Hauser (20). Chemical shifts in parts per million (ppm) downfield from tetramethylsilane, using the terminal methyl peak at 0.90 ppm as an internal standard, were $(CH_2)_n$: 1.30 ppm; CH_2CH_2CO: 1.57 ppm; $CH_2C=C$: 2.02 ppm; and CH_2CO: 2.23 ppm; these were temperature independent within the accuracy of measurement (±0.03 ppm).

Peak areas representing integrated intensities were determined by planimetry on expanded printouts, to an estimated accuracy of ~10% for $T \geq 23°C$ and ~20% for $T < 23°C$. The linewidth (Hz) of the bulk CH_2 was measured digitally on the NMR display as the width at half the peak height.

Electron microscopy

Lipoprotein samples, dispersed in the d 1.006 g/ml saline solution, were shipped in insulated Dewar flasks to Berkeley, where they were immediately transferred to a 37°C incubator. LP was not dialyzed before negative staining. Electron microscopy of samples at 37°C or higher was carried out by maintaining sample, stain, grids, and forceps at the desired temperature by means of a slide warmer. A small droplet of sample was placed on the grid and allowed to remain there for 30 sec. The grid was then stained and washed with approximately 20 drops of 2% sodium phosphotungstate, pH 7.4, at 37°C or 58°C, which rinsed most of the excess salt and protein material present in the sample. Negatively stained samples were immediately examined in a JEOL 100C electron microscope.

To determine the effects of low temperature on lipoprotein structure, the samples were placed in the refrigerator (4°C) for 24 hr. During negative staining for electron microscopy, grids, forceps, stains, and samples were kept at 4°C.

Analytical ultracentrifugation

Lymph chylomicrons and VLDL, which had not been cooled below 23°C, were examined by turbidimetric an-

[1] Hamilton, J. A. Unpublished experiments.

alytical ultracentrifugation using a Beckman Model E ultracentrifuge equipped with a photoelectric scanning system and multiplexer, as described by Ma, Schumaker, and Knobler (21). TG-rich LP from the 8–24 hr lymph collection of Rat 2 was diluted 76-fold with 0.196 molal NaCl (d 1.0063 g/ml) containing Na_2 EDTA (400 mg/l), NaN_3 (500 mg/l), and gentamycin (50 mg/l), at 23°C. The LP dispersions were spun in a Beckman AnD rotor at 3000 rpm (VLDL) or 1500 rpm (chylomicrons), first at 23°C and then at 17.5°C, in order to observe any differences in the flotation rates at the two temperatures. Absorbance at 330 nm along the cell was monitored at 8-min intervals.

RESULTS

Lipid composition of lymph chylomicrons and VLDL

Triglycerides comprised 84.5–86.5% of the chylomicron lipid mass in both 0–8 hr and 8–24 hr collection periods in both rats, and phosphatidylcholine comprised 10.4–12.2%. Free and esterified cholesterol, sphingomyelin, and phosphatidylethanolamine were present in small amounts (0.4–1.3%). Fatty acids, partial glycerides, and lysolecithin were not detectable, even after storage for more than 3 months at 37°C. VLDL was similar to chylomicrons except that less TG (71–81%) and more phosphatidylcholine (14.7–23.8%) were present. In both chylomicrons and VLDL, 58–72% of TG fatty acids were palmitate and 6–16% were stearate. For the eight LP samples examined, TG-fatty acids averaged 74 ± 1% saturated (**Table 1**).

Differential scanning calorimetry

On first heating lymph chylomicrons and VLDL from 37°C to 60°C, no enthalpy changes were observed. Thus, the LP lipids were in a liquid state when secreted. A typical experiment appears in **Fig. 2A** and all eight lipoprotein samples showed generally similar patterns.

During cooling from 60°C at 5°/min, the LP samples began to crystallize at around room temperature (T_c range 17–22°C, **Table 2**). Little change in the crystallization onset (T_c) or the peak (T_p) temperatures occurred after the LP was denatured by heating to 90°C. In contrast, lipids isolated from LP began to crystallize at temperatures 10°C higher than within the LP, suggesting that the size, shape, or protein component of the LP favored undercooling of the LP lipid.

When LP or denatured LP samples were reheated from −3°C to 60°C, each showed a single major melting transition. The melting onset temperatures (T_o) ranged between 11–32°C, and melting was complete between 45–64°C in individual samples (**Table 3**). This major transition corresponded to the second endothermic transition seen in the extracted lipids, which melted over a similar temperature range (Table 3 and Fig. 2B). Heating the LP to 37°C after cooling to 0°C melted half or less of the LP-TG that had crystallized at the lower temperature. Over all the LP samples, the melting enthalpies obtained on immediately reheating the LP after cooling to −3°C appeared to correlate with the proportion of saturated fatty acids in the LP-TG (Table 1). There was a positive association between the 16:0/18:1 ratio and ΔH over all the LP samples ($r = 0.67$), which just failed to reach significance at the 95% confidence level (where for $N = 7$, $r = 0.707$). A positive correlation between ΔH and FA saturation in LP-TG would be predicted since the more saturated the TG, the larger should be the fraction of TG that would be crystalline below T_c.

In separate experiments, the kinetics of crystallization of non-denatured chylomicrons were examined by varying the cooling rate and the time of storage at −3°C. When chylomicrons were cooled at 5°/min, 12.4 cal/g

TABLE 1. Triglyceride fatty acid composition and melting enthalpies of lymph chylomicrons and VLDL

Rat	LP[a]	Collection	FA Percent by Weight[b]							Ratio	ΔH[c]
			12:0	14:0	16:0	18:0	18:1	18:2	Other	16:0/18:2	
		hr									*cal/g TG*
1	Chylo	0–8	0.8	1.0	68.1	4.9	18.4	3.5	2.9	19.5	13.8 (1.6)
	Chylo	8–24	0	1.1	72.0	5.7	16.6	1.8	2.9	40.0	15.1 (1.7)
	VLDL	0–8	0	0	60.7	8.8	18.9	3.0	8.7	20.0	4.9 (0.4)
	VLDL	8–24	0	0	72.0	9.2	18.8	0	0		8.8 (1.4)
2	Chylo	0–8	0	1.1	62.4	9.6	17.7	2.9	6.2	21.5	2.8 (0)
	Chylo	8–24	0	1.4	63.5	7.0	20.0	2.2	5.9	28.9	17.7 (0.7)
	VLDL	0–8	0.2	0.9	57.9	10.6	20.7	4.5	5.1	12.9	3.7 (0.1)
	VLDL	8–24	0	0	60.4	16.0	19.2	2.3	2.2	26.3	9.7 (2.1)

[a] Lipoproteins were isolated at 37°C as described in Methods.
[b] TG-FA were analyzed by GLC of methyl esters after TLC separation of TG.
[c] Means (SD) of heating runs at 5°/min from two to six continuous heating-cooling cycles between −3°C and 60°C, at a sensitivity of 0.1–0.2 mcal/sec. Samples were not held at either temperature limit.

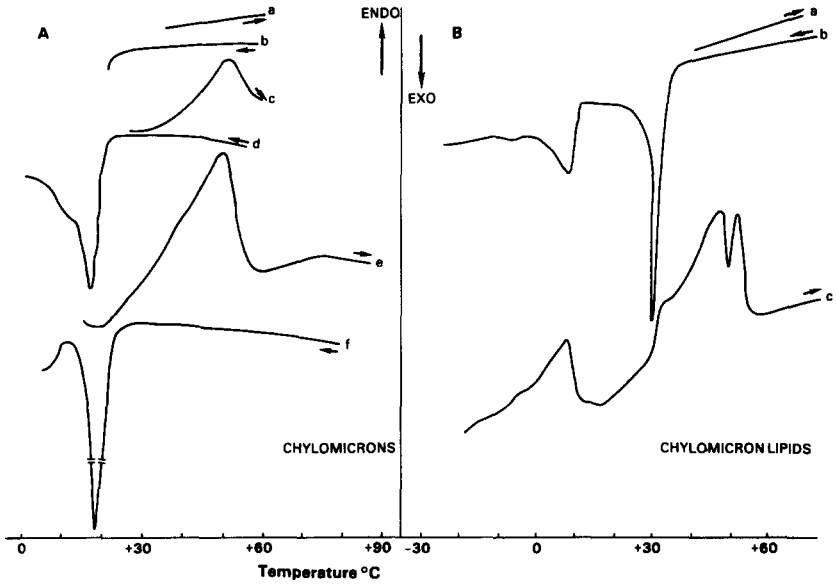

Fig. 2. Differential scanning calorimetry curves of lymph chlyomicrons (A) and of chylomicron lipids (B). Chylomicrons were isolated from the 8–24-hr lymph collection of Rat 2 at 37°C, and scanned at a sensitivity of 0.2 mcal/sec during heating and cooling at 5°/min. A. Chylomicrons containing 2.35 mg TG: a) absence of phase transition between 37–60°C shows liquid state; b) onset of crystallization at 22°C; c) appearance of endothermic phase transition between 30–60°C after cooling to 22°C; d) large exothermic transition due to crystallization between 22–0°C; e) increased endothermic transition between 27–60°C due to melting of large crystalline phase formed between 22–0°C; f) after protein denaturation at 90°C crystallization occurred over a narrower temperature range. B. Chylomicron lipids containing 2.49 mg TG: a) no phase change is observed between 37–60°C when melted lipids are supercooled to 37°C; b) two distinct exothermic transitions are seen; the onset temperature is higher than for chylomicrons although significant undercooling still occurs; c) two phase changes are present; melting is complete at ~60°C.

TG was released during the exothermic liquid-to-crystalline transition. When the cooling rate was slowed to 0.3°/min, the crystallization enthalpy was doubled (25.4 cal/g TG), implying that twice as much TG had crystallized during the slower cooling rate. Holding the LP at −3°C produced further crystallization. When chylomicrons were held at −3°C for 18 hr and then reheated at 5°/min, the melting enthalpy reached 48.5 cal/g TG,

TABLE 2. Crystallization onset (T_c) and peak (T_p) transition temperatures during cooling of melted lymph lipoproteins and lipoprotein lipids[a]

			Chylomicrons[b]		VLDL[b]	
	Rat	Collection	T_c	T_p	T_c	T_p
		hr		*°C*		
Lipoprotein	1	0–8	18 (2)	7 (1)	17 (1)	10 (0)
	1	8–24	22 (0)	14 (3)	19 (2)	6 (1)
	2	0–8	19	ND	17 (1)	<0
	2	8–24	22 (0)	16 (3)	21 (2)	15
Heat-denatured lipoprotein[c]	1	0–8	20 (1)	13 (0)	20	13
	1	8–24	20 (4)	17 (0)	19	14 (0)
	2	0–8	15 (0)	10 (0)	17 (4)	8 (0)
	2	8–24	21 (2)	16 (0)	21 (2)	16 (0)
Extracted lipid[d]	2	0–8			28 (0)	18;4
	2	8–24	32 (1)	29 (0); 6 (0)		

[a] No transitions were observed when lipoproteins, isolated at 37°C, were heated and cooled between 37–60°C.
[b] Lipoproteins containing 1.3–4.1 mg TG were cooled to −3°C and lipids were cooled to −23°C, at 5°C/min at a sensitivity of 0.1–0.2 mcal/sec.
[c] Lipoproteins were denatured by heating to 90°.
[d] Two distinct transitions were observed, unlike lipoproteins, as shown in Fig. 2, Chylomicron TG 2.5 mg; VLDL TG 4.2 mg.
Data are means (SD) of one to four cooling runs; ND, not determined.

TABLE 3. Temperature limits and peak temperatures for endothermic melting transitions of lymph lipoproteins and lipoprotein lipids[a]

	Rat	Collection	Chylomicrons[b] T_o	Chylomicrons[b] T_m	Chylomicrons[b] T_p	Chylomicrons[b] T_e	VLDL[b] T_o	VLDL[b] T_m	VLDL[b] T_p	VLDL[b] T_e
		hr					°C			
Lipoprotein	1	0–8	21 (7)	43 (1)	40 (1)	60 (6)	17 (4)	37 (1)	40 (1)	47 (2)
	1	8–24	21 (5)	49 (5)	51 (1)	62 (2)	20 (1)	40 (0)	40 (1)	60 (4)
	2	0–8	32	39	37 (0)	57	20 (1)	36 (0)	37 (0)	45 (1)
	2	8–24	31 (3)	48 (2)	52 (1)	60 (1)	30 (1)	41 (0)	50 (2)	60 (0)
Heat-denatured lipoprotein[c]	1	0–8	19	41	45	55	ND	ND	ND	ND
	1	8–24	13	47	51	64	19	43	45	53
	2	0–8	32	39	37	58	17	37	38	48
	2	8–24	28	46	50	60	11	43	47	58
Extracted lipid	2	0–8					−18; 26[d] (2) (1)	2; 35 (2) (1)	7; 38 (0) (0)	26; 47 (1) (1)
	2	8–24	−9; 29 (1) (1)	5; 43 (1) (0)	7; 48 (0) (0)	11; 55 (1) (1)				

[a] T_o and T_e define the start and end of the melting transition, T_m defines the temperature at which the lipids are half melted, and T_p defines the peak of the melting transition, as illustrated in Fig. 1.
[b] Lipoproteins and lipids were heated at 5°/min from −3°C (LP and denatured LP) or from −23°C (lipid) at a sensitivity of 0.1–0.2 mcal/sec.
[c] Lipoproteins were denatured by heating to 90°C.
[d] Two transitions were observed, unlike lipoproteins, as shown in Fig. 2.
Data are means (SD) of one to four heating runs; ND, not determined.

similar to the melting enthalpy (beta to liquid) for tripalmitin (53.1 cal/g) and tristearin (54.5 cal/g).

Overall, the data imply that the native LP was secreted with the lipids present as undercooled liquids at 37°C. When cooled to around 0°C, about half of the component TG crystallized rather rapidly; rewarming the LP to 37°C melted about half of the crystallized TG, equivalent to only about 75% liquid after cooling and rewarming. Heating to above the transition temperature (i.e., to 55–60°C) was necessary to melt all the TG once the LP had been cooled to 0°C. When LP was held at about 0°C for 18 hr, crystallization continued until possibly 90% of the TG had crystallized.

X-ray diffraction

To investigate the structural organization of the triglycerides in intact chylomicrons, x-ray diffraction studies were carried out at specific temperatures previously shown by calorimetry to produce a stable lipid state following a phase transition. Representative diffraction patterns obtained from chylomicrons of the 8–24 hr lymph collection from Rat 2 are illustrated in **Fig. 3.** At 37°C, immediately following isolation and concentration at the same temperature, the x-ray diffraction pattern exhibited a broad diffuse scattering, centered at 1/4.5 $Å^{-1}$ and superimposed on a steeply increasing background due to water scattering. This diffuse scattering at 1/4.5 $Å^{-1}$ is typical of triglycerides in the liquid state.

After rapid cooling to 0.8°C, a diffraction at 1/4.2 $Å^{-1}$, corresponding to the alpha polymorphic form of TG (22), became the dominant feature of the diffraction pattern. Three orders of long spacing corresponding to 43 Å were also observed at 0.8°C. On reheating to 20°C, the pattern changed to one exhibiting sharp diffractions at 1/4.6 $Å^{-1}$ and 1/3.8 $Å^{-1}$ in the wide angle region, together with three well-resolved orders of long spacing at 41 Å in the low angle region. These spacings, particularly those in the wide angle region, are typical of TG in the beta polymorphic crystalline form (22). Always discernable at 1/4.5 $Å^{-1}$, however, was a broad diffuse scattering suggesting a substantial residual liquid component. Heating to 32°C and 37°C produced no further changes in the diffraction pattern. Following heating to 60°C (the end point of the endothermic transition observed on heating in the calorimeter), the diffraction pattern once again transformed to that typical of liquid triglyceride. Subsequent rapid recooling to 11°C resulted in crystallization into the alpha polymorphic form, identifiable by a sharp diffraction maximum at 1/4.2 $Å^{-1}$. Again a substantial liquid component was observed at this temperature.

Kinetic aspects of the crystallization of chylomicron TG were followed in an experiment in which the chylomicron sample was cooled rapidly from the liquid state at 60°C to 1°C. Subsequent x-ray diffraction patterns were recorded at 30-min intervals with the sample held isothermally at 1°C (data not shown). Initial crystallization occurred into the alpha polymorphic form (diffraction maximum at 1/4.2 $Å^{-1}$), which slowly transformed into the beta polymorphic form (sharp diffractions at 1/4.6 and 1/3.8 $Å^{-1}$); by 7 hr no alpha form remained. At all times a liquid component was also present at 1/

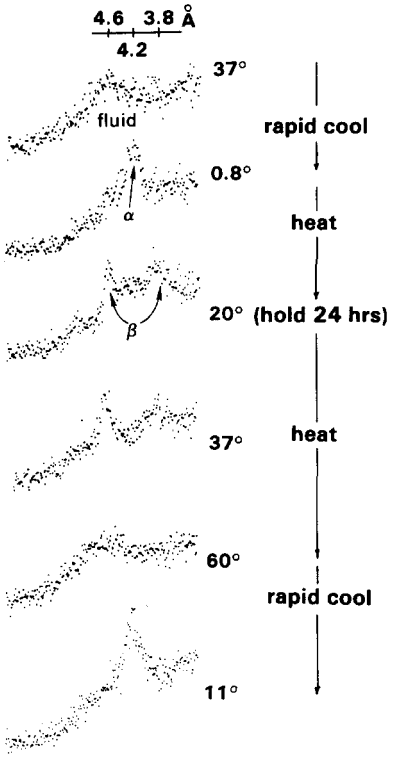

Fig. 3. Wide angle x-ray diffraction patterns of lymph chylomicrons as a function of temperature. Chylomicrons were isolated from the 8–24 hr lymph collection of Rat 2 at 37°C, as described in Methods. Representative diffraction patterns, beginning at 37°C, are shown at the indicated temperatures. Both alpha and beta polymorphic forms may be seen with short spacings of 4.2 Å and of 4.6 and 3.8 Å, respectively, superimposed on a large liquid phase present at all temperatures studied. Upon rapid cooling from 37°C only the alpha form is seen initially, which converts to beta during storage in the cold.

4.5 $Å^{-1}$. These studies suggest that the stable beta TG polymorph of the native LP can be significantly undercooled, and that the chylomicron TG first crystallizes into the alpha polymorph which transforms slowly to the stable beta form. A summary of the x-ray diffractions at long and short spacings appears in **Table 4.**

For comparison with whole LP, the x-ray diffraction pattern of the lipids isolated from lymph VLDL is shown in **Fig. 4.** The liquid pattern seen at 60°C persisted as the lipids were cooled rapidly to 37°C. On further cooling to 26°C, the beta polymorphic form appeared with wide angle spacings at 1/4.6 $Å^{-1}$. At −4°C, both alpha and beta forms were seen. On reheating to 23°C or to 37°C, the alpha form disappeared but the beta polymorph remained present, not melting until 55°C. The alpha to beta transition, which occurred close to 23°C, probably was responsible for the marked enthalpy change observed by DSC at the same temperature (see Fig. 2B). Holding the LP lipids at −10°C for 16 hr did not appreciably alter the proportion of alpha and beta forms seen when the lipids were first cooled to −4°C. This contrasts with the results obtained with whole LP, where the alpha form slowly disappeared at low temperatures. The x-ray diffraction data thus also suggest a constraining influence of the LP size, shape, or protein component on the physical properties of the LP lipids.

NMR

Proton NMR spectra of VLDL from the 8–24 hr lymph collection of Rat 2 were obtained sequentially at 38.5, 31.5, 26, 23, 18.5, 14.5, 13, and 8°C. The LP sample was then cooled in ice-water for 10 min and additional spectra were obtained at 23°C and 41°C. After heating to ~50°C for 15–20 min, the sample was stored at 37°C for 6 days and a final spectrum was obtained at 40°C under the previous conditions. This spectrum was identical in peak intensities and linewidths to the original spectrum obtained at 38.5°C.

A second series of temperature-dependent spectra was obtained one week later in the sequence 36, 31, 26.5, 23.5, 18.5, 16.5, 13, 10.5, 7.5, 0°C for 15 min, then 7.0, 15, 19, 22.5, 35.5, and 44°C. The aliphatic region of selected spectra obtained during cooling is shown in **Fig. 5.** For all peaks the linewidths increased and the intensities decreased markedly as the temperature was lowered through the thermal transition observed by DSC and x-ray diffraction when the LP was cooled (see Figs. 2A and 3). The intensities of the allylic peaks changed less with temperature than the intensities of α CH_2 peaks (see spectral inserts with higher vertical expansion, Fig. 5).

The integrated intensities for proton peaks at different locations along the fatty acyl chains (α CH_2 protons, allylic protons, and methyl protons) during the two heating and cooling runs are shown as a function of temperature in **Fig. 6A, B, C.** Agreement between the two trials was within the experimental measurement error of 10–20%. The intensities at any given temperature differed with the thermal history. During cooling all three intensities were constant between 38°C and 25°C and then decreased rapidly, while during heating they increased linearly between 8°C and ~45°C. At ~45°C the peak intensities were 80–90% of the original intensities observed at 37°C before the LP were cooled, suggesting that most TG had melted. This agrees with the DSC experiments which also demonstrated ~80% melting of cooled LP at 45°C (see Fig. 2A). The ratio (not shown) of the α CH_2 (Fig. 6A) to the CH_3 (Fig. 6C) peak areas, which represents the relative mobilities of protons at the carbonyl end compared with those at the terminal end of the acyl chains, remained constant during both heating and cooling. These data imply that both ends of the chain crystallized and melted simultaneously.

The temperature-dependent behavior of the allylic peak differed from those of the terminal methyl and the

TABLE 4. Summary of x-ray diffraction data

Temperature	Long Spacings[a]	Short Spacings	Polymorphic Form
°C		Å	
Chylomicrons (Rat 2, 8–24 hr)			
37 (following isolation)	absent	4.5 diffuse	liquid
0.8	43	4.2	α
		4.5 diffuse	liquid
20	41	4.6, 3.8	β
		4.5 diffuse	liquid
37 (following 0.8 and 20)	41	4.6, 3.8	β
		4.5 diffuse	liquid
VLDL lipid (Rat 2, 8–24 hr)			
37 (following 55–60)	absent	4.5 diffuse	liquid
−4	43	4.6, 3.8	β
		4.2	α
		4.5 diffuse	liquid
23	44	4.6, 3.8	β
		4.5 diffuse	liquid
37 (following −4)	~48	4.6, 3.8	β
		4.5 diffuse	liquid

[a] The accuracy of the long spacing data was limited by the resolution of the x-ray detection system. Values are reported primarily to document further the observation of the sharp diffraction observed indicative of crystallization.

α methylene peaks. On cooling the LP from 37°C to 10°C, the CH_3 and α CH_2 peak areas decreased by 60–80% (Fig. 6C and 6A) while the allylic peak was only halved (Fig. 6B), implying that relatively more of the saturated acyl chains were immobilized during TG crystallization. The preferential crystallization of the satu-

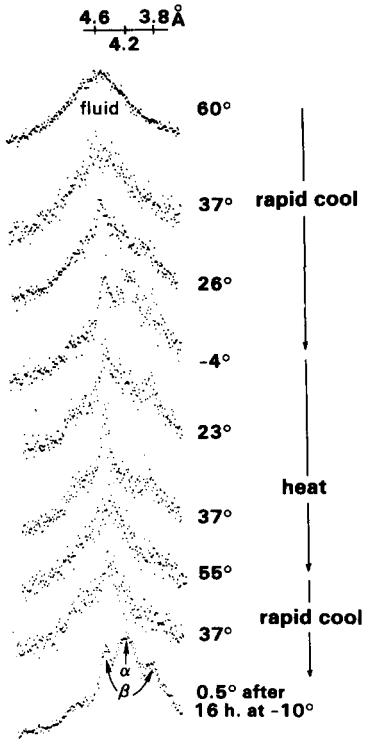

Fig. 4. Wide angle x-ray diffraction patterns of lymph VLDL lipids as a function of temperature. Lipids were isolated from heat-denatured VLDL obtained from the 8–24-hr lymph collection of Rat 2, as described in Methods. Diffraction patterns were obtained sequentially at the indicated temperatures. Unlike the intact LP, both alpha and beta polymorphs are present upon rapid cooling and there is no transformation during cold storage.

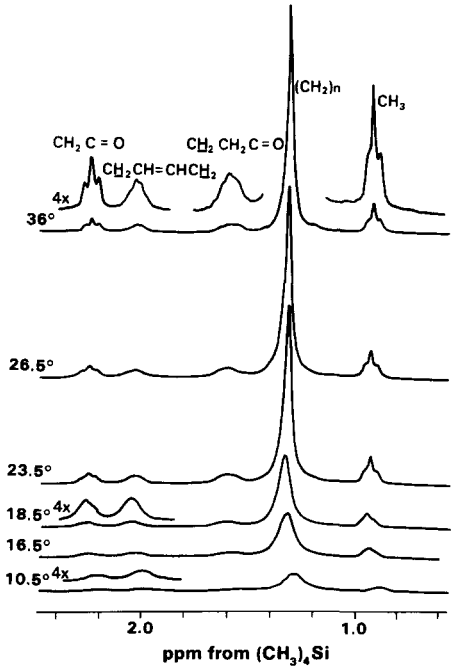

Fig. 5. The effect of cooling from 36°C to 10°C on the proton NMR spectrum of lymph VLDL. VLDL was isolated from the 8–24-hr lymph collection of Rat 2, as described in Methods. Starting with the lipoprotein in the native state at 36°C, spectra were recorded at decreasing temperatures down to 10.5°C. Each spectrum is the resultant of ten spectral accumulations obtained with a pulse interval of 4.0 sec, 8192 time domain points, and 2000 Hz spectral width. Chemical shift assignments were CH_3: 0.9 ppm; $(CH_2)_n$: 1.30 ppm; $C\underline{H}_2CH_2CO$: 1.57 ppm; CH_2C = C: 2.02 ppm; CH_2CO: 2.23 ppm. Instrumental conditions were matched for all spectra; inserts show selected regions at 4-fold vertical expansion.

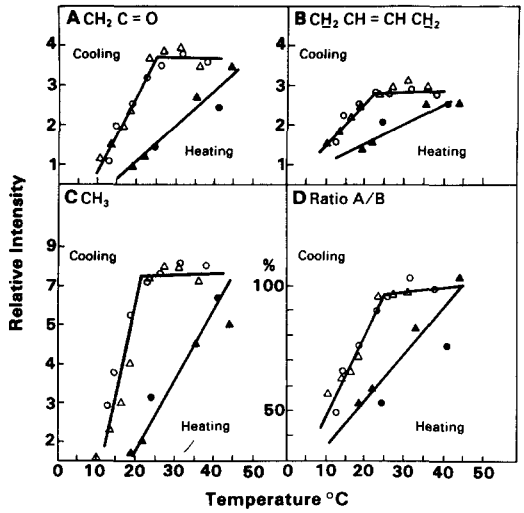

Fig. 6. Intensities of individual peaks in the proton NMR spectrum of lymph VLDL, illustrating supercooling. A: Intensity of methylene protons as a function of temperature. B: Intensity of methylene protons alpha to olefinic double bonds. C: Intensity of terminal methyl protons. D: Ratio of intensities of A/B expressed as a percentage of the ratio in the melted state. The rapid decrease in ratio below 25°C implies that, on cooling, the carbonyl region of the acyl chains, representative of both saturated and unsaturated fatty acids, is more immobilized than the central olefinic region of the unsaturated fatty acids. Symbols used: cooling, ○, △; run I, ○, ●; heating, ●, ▲; run II, △, ▲.

rated chains is further emphasized in Fig. 6D, which shows the ratio of the α CH_2:allylic peak intensities as a function of temperature. This ratio would be constant (100%) at all temperatures if saturated and unsaturated chains crystallized and melted simultaneously. However, the ratio decreased on cooling from 25°C to 8°C although it was fairly constant from 40°C to 25°C. Thus, TG that remained fluid at low temperatures was less saturated than the total TG of the LP.

Although all the aliphatic peaks (as well as glycerol and olefinic peaks not shown) clearly broadened with decreasing temperature, only the bulk CH_2 peak could be measured with sufficient accuracy for quantitation. Fig. 5 and **Fig. 7** show that this linewidth was narrow at 37°C, remained constant when the LP cooled from 37°C to ~25°C, and increased rapidly below ~25°C. On heating from 8°C, the linewidth decreased almost linearly with temperature. After heating to ~50°C followed by storage at 37°C (not shown), the linewidth at 41°C was similar to the initial value at 37°C.

The time dependence of the integrated intensities during longer storage at low temperature was studied after concentrating the VLDL sample ~twofold. **Fig. 8** shows selected spectra from a series obtained at 37°C, followed by rapid cooling to 4°C (time zero), with subsequent spectra obtained at 6–7°C as a function of time (0.15, 0.5, 1, 2, 8, and 24 hr). A final spectrum was obtained immediately on reheating to 37°C. The integrated intensities of all the peaks decreased rapidly after cooling to 6–7°C with little further change after 8 hr. The areas of the bulk CH_2 peak as a function of time, normalized to native uncooled LP at 37°C, are shown in the insert in Fig. 8. The final spectrum, obtained at 37°C after the LP was held at 6–7°C for 18 hr, shows that the intensities of all peaks were lower than in the initial 37°C spectrum; integration of the bulk CH_2 peak area indicated that about 25% of the TG had not melted. This is similar to the results for other resonances (Fig. 6A, B, C) and corroborates the data obtained by DSC and x-ray diffraction experiments. As shown in Fig. 8, the LP solidified at 6–7°C with a half-time of less than 0.15 hr. Ultimately, 70–75% of the TG solidified at that temperature, leaving 25–30% liquid.

Electron microscopy

Changes in chylomicron morphology as a function of temperature are shown in representative electron micrographs in **Fig. 9** and are summarized in **Table 5.** Chylomicrons maintained at 37°C were round particles, extremely heterogeneous in size (Fig. 9A). After incubation at 4°C, 20–30% of the particles appeared flattened and polygonal (Fig. 9B). However, after incubating the chylomicrons at 58° for 10 min the lipoprotein profile was again round (Fig. 9D) and the size distribution was similar to that of the native particles (Table 5).

VLDL exposed to low temperatures (not shown) displayed structural alterations similar to chylomicrons. At 4°C many particles became flattened and polygonal and this morphology could not be completely reversed by subsequent incubation at 37°C. Following a 10-min incubation at 58°C, however, the VLDL again appeared

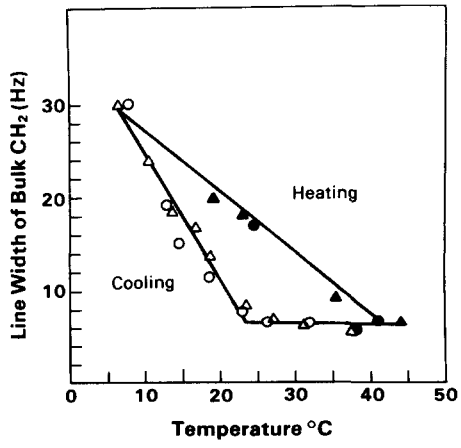

Fig. 7. Line widths ($V_{1/2}$) of bulk methylene protons as a function of temperature. Two separate NMR experiments are illustrated, with symbols as in Fig. 6.

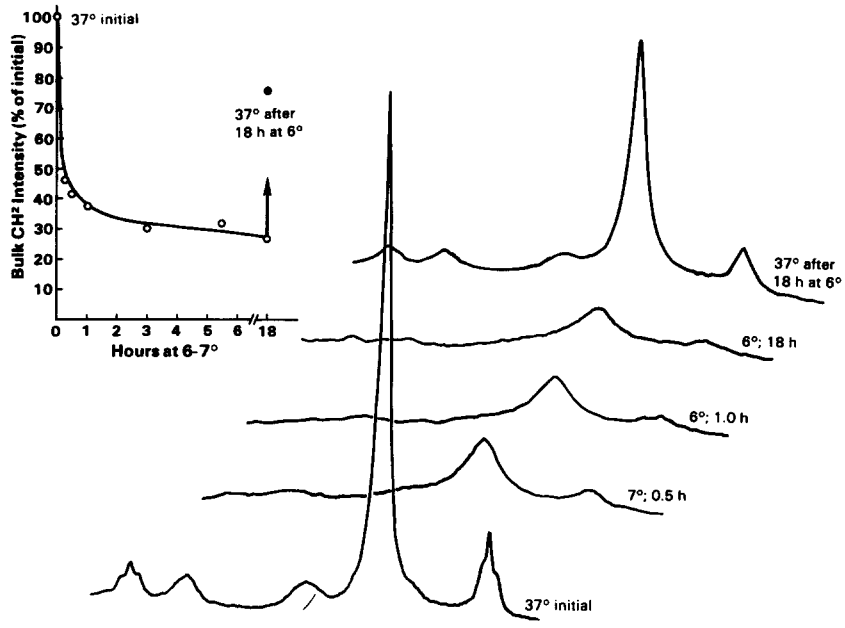

Fig. 8. Effect of storage at 6–7°C on the NMR spectrum of lymph VLDL. Lymph VLDL was isolated from the 8–24-hr lymph collection of Rat 2. The figure shows progressive loss of mobility with time of storage at low temperature and incomplete melting at all frequencies on reheating to 37°C.

spherical with a similar size distribution to the original VLDL. There was no evidence that heating LP to 58°C produced significant coalescence of the particles.

Analytical ultracentrifugation

Turbidimetric ultracentrifugation of lymph LP was performed first at 23°C, just above the TG crystallization temperature (Table 2), and then at 17.5°C, where about half the TG was crystalline (See Fig. 6). Previous studies by DSC (see Fig. 2A) revealed that cooling the LP from 23°C to 17°C was accompanied by a large exothermic (liquid to crystalline) phase change. Based on a TG content equal to 70% of the VLDL mass, a crystalline TG density 12% higher than that of liquid TG (23), and 50% of TG crystallized at the lower temperature, the density of the VLDL particles should have been approximately 4% higher at 17.5°C than at 23°C. The results of the first five scans at each temperature for rat lymph VLDL, taken 8 min apart, are shown in **Fig. 10.** There was a dramatic decrease in the rate of flotation of VLDL particles at 17.5°C compared with 23°C, reflecting the greater density of the VLDL at the lower temperature. Qualitatively similar results were obtained for chylomicrons (data not shown).

DISCUSSION

Recent discovery of lipid phase transitions in cholesterol ester-rich lipoproteins (1) indicated that lipids in the core of lipoproteins can behave similarly to bulk lipids. Cholesterol esters of biological origin, which have transitions around body temperature (1, 2, 6), display similar but not identical transitions when located in the core of small lipoprotein particles (2, 6). We wished to understand the physical state of the triglycerides located in the core of triglyceride-rich particles synthesized by the intestine, and how temperature affected the lipoprotein structure and properties. We have examined triglyceride-rich intestinal lipoproteins obtained from rats fed a diet rich in palmitic acid. The triglycerides of these lipoproteins contained about 74% saturated fatty acids, primarily palmitic acid. It is known that neat triglycerides containing a high content of palmitic acid have melting points considerably above body temperature (8, 9, 22, 24). Recently it was shown that highly saturated VLDL secreted by isolated rat livers during perfusion with palmitate displays a major transition between 0–20°C (25).

Puppione et al. (15) described triglyceride-rich particles, isolated from bovine plasma and lymph, that contained a high proportion of saturated fatty acid triglycerides, were too dense, were irregularly shaped, and that scattered light anomolously. It was suggested that these particles might contain crystalline triglycerides. Subsequently we demonstrated that crystalline triglycerides were present (26). Furthermore, triglyceride-rich particles from calf lymph, which contain highly saturated triglycerides, are spherical and float as VLDL when iso-

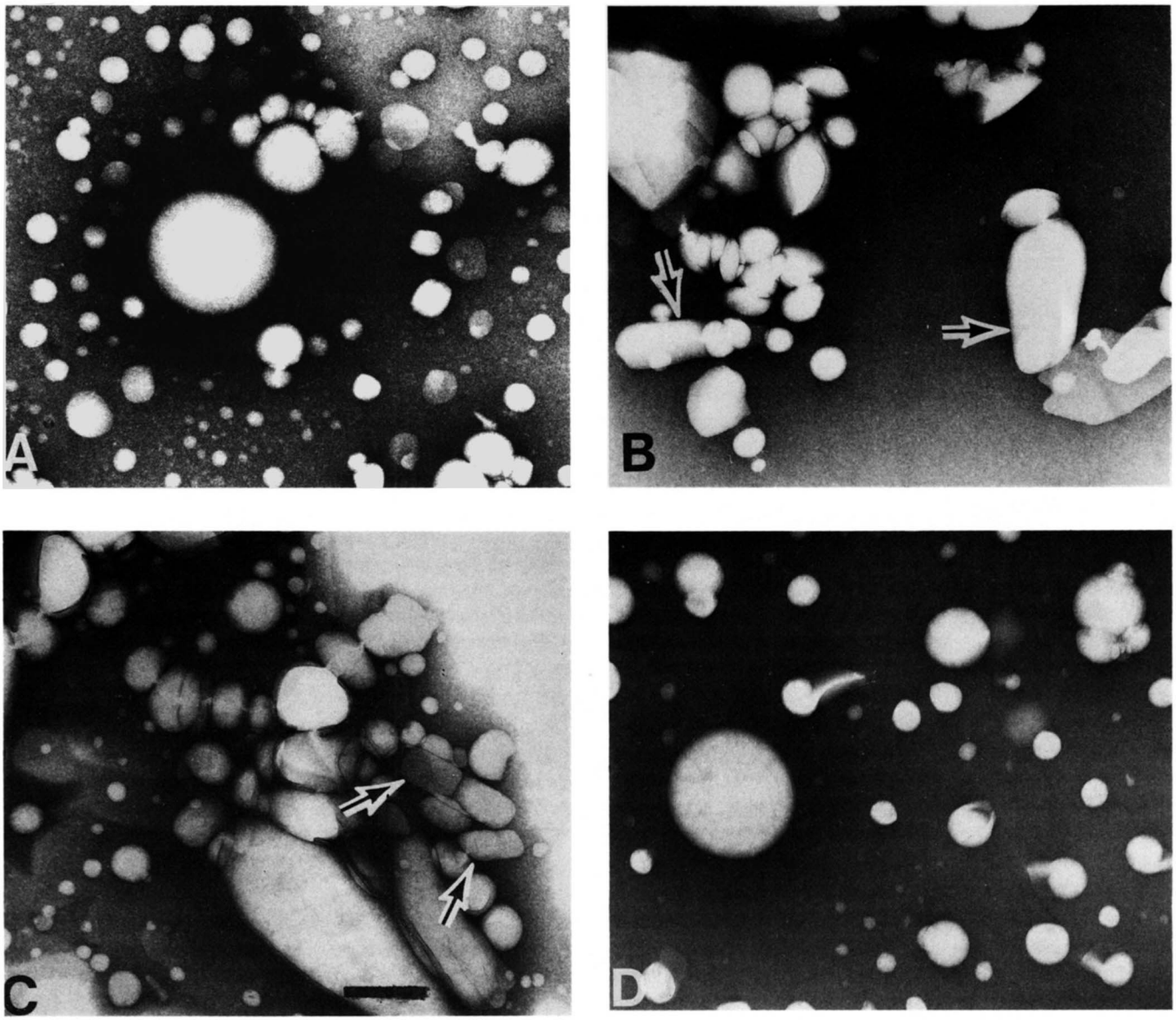

Fig. 9. Chylomicron morphology as a function of temperature. Chylomicrons were isolated at 37°C and were negatively stained as described in Methods. A, Freshly isolated at 37°C. Chylomicrons have round profiles and are extremely heterogeneous in size (see Table 5). B, After storage at 4°C for 24 hr, many particles became flattened and polygonal in shape as indicated by arrows. C, Heated to 37°C after 24 hr at 4°C. Persistence of polygonal particles is evident. D, Heated to 58°C for 10 min. Particles are again round in profile and are similar to the starting sample.

lated at 37°C, but become angular and more dense when cooled to 16° (26). Thus, it was suggested that cooling this VLDL to 16°C could initiate crystallization of the triglyceride and alter the shape and density of the particles.

The present report describes an in depth study of chylomicrons and VLDL isolated from intestinal lymph of rats fed a diet designed to enrich these lipoproteins with saturated fatty acids. The lipoproteins were isolated and maintained at 37°C so that they could be characterized first in their nascent state; the effects of changing the temperature were then evaluated. Chylomicrons and VLDL secreted by the intestine differed in their mean size but the fatty acid composition of the triglycerides was similar, with 74% of TG-FA saturated. The final melting point of the triglycerides isolated from these lipoproteins was 48–55°C, similar to that of the lipoproteins themselves (56 ± 6°C; $\bar{x}$ ± SD). A variety of physical techniques was used to study the physical properties of the lipoproteins. Differential scanning calorimetry

TABLE 5. Chylomicron and VLDL size and shape as a function of temperature

Temperature	Polygonal			Spherical	
	Long Axis	Short Axis	N[a]	Diameter	N[a]
°C	Å			Å	
Chylomicrons					
A 37, freshly isolated				746 (344)[b]	200
B 4	3514 (1342)	1416 (624)	30	755 (345)	110
C 37 after 4	3009 (709)	1103 (330)	30	759 (268)	100
D 58 after 4				724 (289)	100
VLDL					
A 37, freshly isolated				594 (193)	200
B 4	1827 (604)	897 (297)	39	567 (175)	100
C 37 after 4	2095 (664)	918 (249)	50	601 (218)	200
D 58 after 4				622 (242)	200

[a] Number of particles measured.
[b] Mean (SD).
Lipoproteins were negatively stained and examined at the temperatures indicated, and were photographed and measured at a magnification of ×60,000. Approximately 20–30% of the particles appeared polygonal under conditions B and C. More precise determination of the proportion of irregularly shaped LP was not possible due to clumping of the particles on the grids.

determined the temperature ranges and quantitated the enthalpies (ΔH) of the phase transitions. X-ray diffraction experiments defined the polymorphic forms of the crystalline material. NMR experiments determined the quantity of crystalline material present and the chemical type of the fatty acid molecules undergoing crystallization. Supporting evidence was obtained from electron microscopy, which was used to describe the sizes and shapes of particles, and from analytical ultracentrifugation, which defined their densities. The thermal histories of the particles were monitored meticulously so that in all experiments appropriate thermal histories were compared.

Our experiments have demonstrated that native VLDL and chylomicrons as isolated from lymph at ~37°C behave similarly and that their triglycerides are in the liquid state supercooled 0–15°C below their peak melting points. The particles are spherical and the particle density is appropriate for triglycerides in the liquid state. When the lipoproteins are cooled, crystallization is initiated at about 22°C, first to the alpha form of triglyceride which later undergoes a polymorphic transition to the stable beta form. When cooled very slowly to <7°C, or stored at laboratory coldroom temperatures (0–4°C), 75% or more of the triglyceride crystallizes in the beta polymorphic form, which distorts the normally spherical particle to angular, non-spheroidal shapes. Approximately 25% of the triglycerides remains in the liquid state at 6–7°C and these triglycerides are enriched in fatty acids with double bonds. When the partially crystalline particles are reheated, they undergo a gradual transition to the liquid state between about 20°C and about 60°C, with a peak temperature at 44 ± 6°C. Cooled particles reheated only to 37°C still contain approximately 25% crystalline triglyceride. Thus, nascent triglyceride-rich lipoproteins containing highly saturated triglycerides are in a metastable, undercooled liquid state and isolation at temperatures below about 25°C causes partial crystallization of the lipoprotein triglycerides. Reheating to body temperature does not melt all of the crystallized triglyceride or restore the lipoprotein to its native state. Heating to 58°C causes complete melting

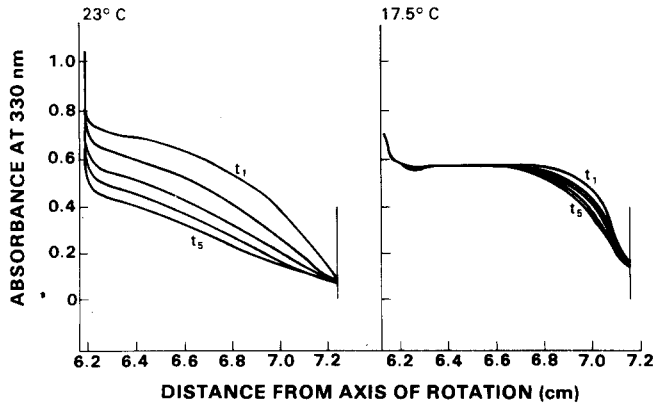

Fig. 10. Analytical ultracentrifugation of lymph VLDL as a function of temperature. VLDL was isolated from the 8–24-hr lymph collection of Rat 2, as described in Methods. Analytical ultracentrifugation was performed in a d 1.0063 g/ml medium, first above the crystallization temperature (23°C) and then after crystallization of about half the TG (17.5°C). The figure, which shows the first five scans at each temperature (t_1–t_5) taken 8 min apart, illustrates the markedly reduced flotation rate at the lower temperature due to the increased density of the partially crystallized lipoprotein.

and forms a particle morphologically similar to the nascent lipoprotein. Cooling from 58°C to 37°C re-establishes a metastable particle.

The implications of these studies are important. Virtually all standard techniques for isolating triglyceride-rich lipoproteins from plasma or lymph include some steps in which the preparation is cooled (e.g., the sample is collected over ice, isolations are carried out at or below room temperature, the samples are stored in a refrigerator). Thus, it is probable that all chylomicrons containing highly saturated triglycerides undergo partial crystallization during their isolation. Crystallization produces particles with abnormal shapes, and, in the case of the bovine lipoprotein (15), this results in the formation of a lipoprotein fraction that isolates in the intermediate density range (IDL). The metabolic fate of such partially crystallized particles when re-injected into animals is not known, but it may differ from that of the native lipoprotein isolated and maintained at 37°C. Florén and Nilssen (27), for example, noted that chylomicrons from butter-fed rats, isolated at low temperatures, behaved differently from chylomicrons isolated from corn oil-fed animals. Lipoprotein lipase appeared to hydrolyze the unsaturated triglycerides preferentially, leaving the saturated triglycerides in the remnant core. This selective lipolysis may have resulted from selective crystallization of the more saturated triglycerides which made them unavailable for the lipase reaction. Florén and Nilsson (27) noted further that more unhydrolyzed triglyceride remained in the liver when chilled chylomicrons from butter-fed rats were injected, compared with chylomicrons rich in polyunsaturated fatty acids. Possibly, chylomicron remnants that contained crystalline triglycerides were taken up by the liver but were not degraded normally. Future studies of chylomicron catabolism may require that the chylomicrons, particularly those obtained from animals fed saturated fat diets, are isolated at temperatures above their triglyceride crystallization temperature. We are at present comparing the metabolism of native chylomicrons isolated at 37°C with that of chylomicrons isolated at lower temperatures, which contain partially crystallized triglycerides and whose apoprotein profiles may differ from those of the nascent particles.[2]

The detailed effects of the degree of fatty acid unsaturation, the fatty acid chain length, or the triglyceride stereoisomerism on lipoprotein transition temperatures are not clearly known. However, we can predict that the transition temperature should be related to the degree of unsaturation. Triglycerides present in normal human VLDL are not highly saturated and do not show triglyceride or cholesterol ester transitions in the range of 10–80°C (28). The VLDL does, however, crystallize at about −10°C and melts between −20°C and 10°C (29). We have also shown that chylomicrons obtained from monkeys fed safflower oil have no transitions when isolated at 4°C.[3] Just how much unsaturation is necessary to suppress the crystallization temperature below 4°C is presently under investigation.[4]

Until all possible effects of cooling LP below their crystallization temperatures on their physical, chemical, and metabolic properties are completely resolved, it seems advisable to perform all maneuvers above the LP crystallization temperature if this is known, or else at 37°C, which may require greater emphasis on sterility during handling. Because this approach is technically cumbersome, it is important to establish methods that will restore chilled particles to their original structure and composition. We have shown that heating to 55–60°C restores the physical properties of chilled particles. Possibly, similar treatment in the presence of cofactors (e.g., reheating chilled lymph to 60°C prior to chylomicron isolation) will also restore their chemical and metabolic properties. Such studies are under active investigation in our laboratory.

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[4] Lymph chylomicrons obtained from rats fed a diet with 63% of the fatty acids saturated also showed transitions around room temperature, although the LP was liquid when secreted, similarly to chylomicrons from rats fed the 75% saturated fatty acids. On cooling from 37°C in the DSC, crystallization began at 20–24°C. On reheating from 2°C, the crystallized lipids in the LP began to melt at 15–23°C, peak melting occurred at 46–50°C and melting was complete at about 60°C. Since diets containing fats which are about 60% saturated are commonly fed in dietary studies and LP separations usually have been performed below 20°C, reevaluation of earlier experiments seems advisable, particularly those reports which appeared to show that dietary TG composition influenced the differential formation and composition of LP classes separated by ultracentrifugation.

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