Very low density lipoprotein. Fate of phospholipids, cholesterol, and apolipoprotein C during lipolysis in vitro

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Abstract In this study we have determined the fate of phospholipids, cholesterol, and apolipoprotein C during lipolysis of rat plasma very low density lipoprotein (rat VLDL). The experiment was carried out in vitro with lipoprotein lipase purified from bovine milk, VLDL labeled with [^{14}C]palmitate, [^{3}H]cholesterol, [^{32}P]phospholipids, and ^{125}I-labeled apolipoprotein C and in plasma-devoid systems. Triglyceride hydrolysis ranged between 0 and 98.6%. [^{32}P]Phospholipids, unesterified [^{3}H]cholesterol, and ^{125}I-labeled apolipoprotein C were removed from the VLDL ($d < 1.019$ g/ml) during lipolysis. About one-third of the [^{32}P]phosphatidylcholine was hydrolyzed to lysolecithin, and was transferred to the fraction $d > 1.21$ g/ml. The other two-thirds of the phospholipids were removed unhydrolyzed, mainly to the fraction d 1.04–1.21 g/ml. With the progression of the lipolysis, unesterified [^{3}H]cholesterol was removed from VLDL at increasing rates, predominantly to the fraction d 1.04–1.21 g/ml. ^{125}I-Labeled apolipoprotein C removed from the VLDL partitioned between the fraction of d 1.04–1.21 g/ml and $d > 1.21$ g/ml. Negative-staining electron microscopy of the fraction d 1.04–1.21 g/ml (containing phospholipids, unesterified cholesterol, and apolipoprotein C) revealed many discoidal lipoproteins. [^{3}H]Cholesteryl esters remained associated with the VLDL even when 70–80% of the triglycerides were hydrolyzed. These observations suggest that during in vitro lipolysis of VLDL, surface constituents leave the lipoprotein concomitantly with the hydrolysis of core triglycerides. The process of removal of surface constituents is independent of the presence of an acceptor lipoprotein and may occur in the form of a surface-fragment particle. —**Eisenberg, S., and T. Olivecrona.** Very low density lipoprotein. Fate of phospholipids, cholesterol, and apolipoprotein C during lipolysis in vitro. *J. Lipid Res.* 1979. **20:** 614–623.

Supplementary key words lipoprotein lipase · triglyceride hydrolysis · [^{32}P]phospholipids · [^{3}H]cholesterol · ^{125}I-labeled apolipoprotein C · low density lipoprotein · high density lipoprotein

The metabolism of very low density lipoprotein (VLDL), a major blood plasma triglyceride-carrying lipoprotein, has been extensively studied in the last decade. Most studies agree that during degradation of VLDL, both core and surface constituents of the lipoprotein are removed from the particles (1–6). Indeed, the calculated core volume and surface area of VLDL particles of different size, density, and weight prepared by a variety of methods (5, 7–9) is in very good agreement with the lipid-core model suggested for VLDL (10, 11). It can therefore be concluded that when small VLDL particles are produced by lipoprotein lipase from larger particles, surplus surface constituents are deleted from the lipoprotein in proportion to the decrease of core volume. Whenever studied, the surplus surface constituents generated during the lipolytic process were found in the plasma and were isolated with HDL, at the density interval of 1.063–1.21 g/ml (1–6, 12).

We have recently demonstrated that surplus surface constituents (phospholipids, apolipoprotein C, and unesterified cholesterol) are also freed from lipolyzed VLDL in the absence of plasma or HDL (13). The experiments were carried out with labeled rat plasma VLDL and recirculating isolated perfused rat heart system. In a previous study, however, it was shown that, under similar conditions, apoC molecules are removed from VLDL during in vitro incubations with a purified lipoprotein lipase and in the absence of plasma (14). It is thus possible that the processes responsible for removal of surplus surface constituents from VLDL are similar when lipolysis is carried out in vivo, with a membrane-supported lipoprotein lipase or with a free enzyme and an in vitro incubation system. In the present investigation, this possibility is further evaluated while using rat plasma VLDL labeled with [^{14}C]palmitate, [^{3}H]cholesterol, [^{32}P]phospholipid, and ^{125}I-labeled apolipo-

Abbreviations: VLDL, very low density lipoprotein(s); IDL, intermediate density lipoprotein(s); LDL, low density lipoprotein(s); HDL, high density lipoprotein(s); ApoC, apolipoprotein C.

protein C, and lipoprotein lipase purified from bovine milk.

MATERIALS AND METHODS

Preparation of lipoproteins and labeled lipoproteins

VLDL (d < 1.006 g/ml) was isolated from rat serum as previously described (15, 16). Male rats of the Hebrew University strain were used. The rats' diet was supplemented with 10% sucrose in their drinking water for 48 hr prior to exsanguination. Blood was obtained from ether-anesthetized rats through the abdominal aorta and serum was separated in the SS-2 Sorval centrifuge. VLDL was isolated by ultracentrifugation using a Beckman ultracentrifuge and the 50Ti rotor at 45,000 rpm for 18 hr. The VLDL was washed once in a NaCl solution of 1.006 g/ml. The VLDL was shown to be free of contamination with plasma proteins and other plasma lipoproteins by the following techniques: lipid and protein composition, immunodiffusion against specific antisera, lipoprotein electrophoresis, and polyacrylamide gel electrophoresis of apolipoproteins (4, 13–16). VLDL thus prepared contained 70–75% triglycerides, 10–12% phospholipids, 9–11% protein, and 5–7% cholesterol.

VLDL labeled biosynthetically with [^{14}C]palmitic acid, [^{3}H]cholesterol, and [^{32}P]phospholipids was prepared likewise from the serum of rats injected with the labeled precursor 1, 6, and 16 hr prior to exsanguination, respectively. The procedures for preparing biosynthetically labeled VLDL are detailed elsewhere (13). VLDL labeled with ^{125}I-labeled apoC was prepared by exchange, as previously described (13, 14). Na^{125}I, [1-^{14}C]palmitic acid and [1α,2α(n)-^{3}H]cholesterol were purchased from the Radiochemical Centre, Amersham, England, and $H_3{}^{32}PO_4$ from the Atomic Energy Commission, Beer-Sheba, Israel.

Lipoprotein lipase

Lipoprotein lipase was purified from bovine milk by affinity chromatography on heparin–Sepharose, as previously described (17). The enzyme was prepared in Umea, Sweden and was shipped in a frozen state to Jerusalem. The activity of the purified enzyme was 384 units/mg protein (17) and it did not change during several months of storage at −20°C. Aliquots (1–10 μl) of an enzyme, thawed at 0–2°C, were used as a source of lipoprotein lipase (see below).

Analytic procedures

Lipoprotein protein was determined by the method of Lowry et al. (18). Lipids were extracted by the procedure by Folch, Lees, and Sloane Stanley (19). Lipoprotein phospholipids and lipoprotein cholesterol were determined after lipid extraction by the methods of Barlett (20) and Chiamori and Henri (21), respectively. Triglycerides were determined by the Autoanalyzer technique (22). Phospholipid classes were separated by thin-layer chromatography using glass plates coated with silica gel G (Merck-Darmstadt, Germany) developed in a solvent system containing chloroform–methanol–H_2O 70:25:4 (v/v/v). Neutral lipids (mono-, di-, and triacylglycerol, and unesterified and esterified cholesterol) were separated using a solvent system of petroleum ether (30–60°C)–diethyl ether–acetic acid 80:20:1 (v/v/v). Lipids were visualized by iodine vapors and identified with the help of reference standards; the lipids were scraped off the plate and assessed for radioactivity content. Radioactivity was determined in a Tricarb liquid scintillation spectrometer No. 3380. Radioactivity associated with ^{125}I-labeled apoC was determined in an Autogamma scintillation spectrometer (Packard, La Grange, Illinois).

Experimental procedures

The incubation mixtures contained rat plasma VLDL (0.4 mg of lipoprotein protein, 2.5–3.0 mg of lipoprotein triglycerides), 0.2 M Tris buffer, pH 8.2, and 4 g% (w/v) of fatty acid-poor bovine albumin (Pentex fraction V powder, Miles Laboratory, Kankakee, IL). Aliquots (1–10 μl) of lipoprotein lipase were added immediately prior to the incubation. Incubations were carried out in 6.5-ml cellulose nitrate ultracentrifuge tubes at 37°C in a thermostated incubator without shaking and at a final volume of 6 ml. The time of incubation varied between 5 and 30 min. Several control incubations without enzyme, and control unincubated samples (without enzyme) were prepared in each experiment and were treated exactly as were samples containing the enzyme. At the end of the incubation period, a 1-ml sample was pipetted to another test tube and mixed with 20 ml of chloroform–methanol 2:1 (v/v). This sample was used to determine the generation of [^{14}C]palmitate during the incubation. Ice-cold concentrated NaCl solution (d 1.1168 g/ml) was added to the other 5 ml in a quantity sufficient to bring the density of the solution to 1.019 g/ml (0.47 M NaCl). The tubes were capped, transferred to a precooled (4°C) 40.3 rotor, and subjected to an 18-hr ultracentrifugation at 40,000 rpm (4°C) in a Beckman ultracentrifuge. VLDL and IDL were isolated together by the tube-slicing technique (23) at density < 1.019 g/ml. The density of the d > 1.019 g/ml infranatant was then adjusted to d 1.040 g/ml with solid KBr and lipoproteins of

TABLE 1. [^{14}C]Palmitate-labeled lipids in lipoprotein fractions after incubation of ^{14}C-labeled VLDL without and with lipoprotein lipase

Lipolysis %	Density g/ml	[^{14}C]Palmitate, % of Total ^{14}C	[^{14}C]Palmitate-labeled lipids, % of Total ^{14}C				
			PL[a]	MG + DG	FA	TG	CE
[b]	<1.019	98.4 ± 0.4	4.8 ± 0.5	2.9 ± 0.2	0.9 ± 0.2	89.4 ± 1.0	1.6 ± 0.3
32.7 ± 3.3	<1.019	66.1 ± 2.0	7.4 ± 0.4	4.3 ± 0.2	6.7 ± 0.4	79.3 ± 1.0	2.2 ± 0.2
	1.019–1.04	2.0 ± 0.8	17.1 ± 2.0	19.3 ± 1.0	5.8 ± 1.9	47.8 ± 3.8	9.7 ± 2.6
	1.04–1.21	2.5 ± 0.3	25.0 ± 2.0	5.9 ± 1.2	62.8 ± 2.6	3.7 ± 1.8	1.2 ± 0.5
	>1.21	29.4 ± 1.1	2.1 ± 0.4	4.3 ± 0.9	90.5 ± 3.3	1.8 ± 0.2	0.3 ± 0.1
71.6 ± 3.4	<1.019	37.6 ± 2.9	9.4 ± 1.0	5.3 ± 0.3	8.9 ± 1.3	72.9 ± 1.8	2.9 ± 0.3
	1.019–1.04	1.8 ± 0.5	26.4 ± 3.9	13.7 ± 1.0	8.5 ± 0.2	33.9 ± 3.0	15.1 ± 5.5
	1.04–1.21	5.0 ± 1.1	22.7 ± 2.8	8.8 ± 1.6	58.5 ± 5.0	6.0 ± 1.0	3.3 ± 0.6
	>1.21	55.6 ± 2.0	1.7 ± 0.6	2.6 ± 0.6	92.6 ± 4.7	2.2 ± 0.3	0.9 ± 0.2

The incubation mixture consisted of [^{14}C]palmitate-labeled VLDL (0.4 mg of protein, 2.5–3.0 mg of triglycerides), 0.2 M Tris buffer, pH 8.2, and 240 mg of fatty acid-poor bovine albumin at a total volume of 6.0 ml. Lipolysis is expressed as percent of labeled palmitate liberated during the incubation from labeled triglycerides, as determined in the incubation mixture. Lipoproteins were separated by ultracentrifugation. Lipids were extracted and separated as described in Methods. The amount of lipoprotein lipase was between 1 and 10 μl, and the time of incubation was 5–30 min. Results are mean ± SE of 4–6 experiments.

[a] Abbreviations: PL, phospholipids; MG, monoglycerides; DG, diglycerides; FA, fatty acids; TG, triglycerides; CE, cholesteryl esters.

[b] Sample was incubated at 37°C for 30 min without enzyme followed by ultracentrifugation at d 1.019 g/ml. Similar results were obtained with samples incubated for other time intervals (5–60 min). The distribution of [^{14}C]palmitate-labeled lipids in unincubated ^{14}C-labeled VLDL was essentially identical to that shown in the table.

d 1.019–1.040 g/ml were isolated after a 24-hr centrifugation. Lipoproteins of d 1.040–1.210 g/ml and the residual protein fraction of d > 1.210 g/ml were separated similarly after a 44-hr centrifugation at a salt density of 1.210 g/ml. Lipids in each of the four density fractions were extracted with 20 volumes of chloroform–methanol 2:1 (v/v). The lipid extract was assessed for total lipid radioactivity and for radioactive phospholipids and neutral lipid classes separated as described above. In experiments where [^{125}I]apoC-labeled VLDL was used, the ^{125}I radioactivity was determined prior to lipid extraction and the resulting cpm were corrected for absorption of radioactivity emission by concentrated salts as previously described (24).

The recovery of labeled lipids, after the centrifugation, was complete and ranged between 88 and 104% for [^{14}C]palmitate-labeled lipids, 91–107% [^{32}P]phospholipids, and 94–105% for [^{3}H]cholesterol. Similarly, the recovery of ^{125}I-labeled apoC was 86–103%.

RESULTS

[^{14}C]Palmitate-labeled VLDL

Two examples of the effects of lipolysis on [^{14}C]-palmitate-labeled lipids in VLDL are shown in **Table 1.** Generation of [^{14}C]palmitic acid and transfer of the fatty acids from the VLDL and IDL density (d < 1.019 g/ml) to albumin (d > 1.21 g/ml) is evident. There was a very good agreement between the degree of lipolysis calculated from thin-layer chromatography of the incubation mixture (first column, percent lipolysis) and that calculated from the ultracentrifugal analysis. There was also evidence for accumulation of small amounts of lipolytic products (mono- and diacylglycerols and fatty acids) in the VLDL and IDL density range. Phospholipids, mono-, and di-, and triacylglycerols and cholesteryl esters were the predominant labeled lipids isolated with the fraction d 1.019–1.014 g/ml, and phospholipid and fatty acids of that isolated at d 1.04–1.21 g/ml.

^{32}P-Labeled VLDL

With the progression of lipolysis, ^{32}P-labeled phospholipids were removed from the VLDL and IDL density range and were found to a varying degree in all density fractions (**Fig. 1**). The disappearance of phospholipids showed two components, a slow component at 0–50% lipolysis, and a fast component with continued lipolysis. Two examples of the distribution of radioactivity among phospholipids isolated with the different density fractions are shown in **Table 2.** Lecithin was the predominant labeled phospholipid in the fractions d < 1.019 g/ml, d 1.019–1.04 g/ml, and d 1.04–1.21 g/ml, and lysolecithin in the fraction d > 1.21 g/ml. Small amounts of [^{32}P]lecithin were also found in the fraction d > 1.21 g/ml. [^{32}P]Sphingomyelin constituted 6.3% of the radioactive phospholipids in VLDL. With lipolysis, the ratio of [^{32}P]-sphingomyelin to [^{32}P]lecithin in VLDL (d < 1.019 g/ml) and the fraction d 1.019–1.04 g/ml increased

from 0.076 to 0.097 and 0.115, respectively. However, the ratio was highest in the fraction d 1.04–1.21 g/ml and was 0.172 at 35.8% lipolysis and 0.213 at 86.5%.

Approximately one half of the [^{32}P]lecithin removed from VLDL to fractions of density greater than 1.04 g/ml was found in [^{32}P]lysolecithin. In a separate experiment, the fraction d 1.04–1.21 g/ml was isolated and reincubated with the enzyme. The [^{32}P]lecithin recovered in the fraction d 1.04–1.21 g/ml was almost not susceptible to the phospholipase activity of the enzyme (**Table 3**). It therefore seemed that the primary site of lecithin hydrolysis was at the surface of the VLDL particles, and that the lysolecithin was subsequently transferred to the fraction d > 1.21 g/ml where, presumably, it was bound to albumin. According to this hypothesis, the further removal of the unhydrolyzed phospholipid fraction, now relatively enriched with sphingomyelin, occurred after the initial phase of lysolecithin formation.

[^{32}H]Cholesterol-labeled VLDL

After 30–60 min of incubation of [^{3}H]cholesterol-labeled VLDL in the buffered albumin without enzyme, about 9.5% of the labeled cholesterol was removed from the VLDL and was recovered at the fraction d 1.04–1.21 g/ml (**Table 4**).[1] It was predominantly in unesterified form. With lipolysis, and even when 50–60% of the triglycerides were hydrolyzed, only small amounts of [^{3}H]cholesterol disappeared from VLDL (**Fig. 2**). It was found predominantly in the fraction d 1.04–1.21 g/ml. With continuation of the lipolytic process however, [^{3}H]-cholesterol was removed at an accelerated rate, and was found in the fractions d 1.019–1.04 g/ml and d 1.04–1.21 g/ml (Fig. 2). Labeled unesterified cholesterol and cholesteryl esters were separated in all experiments and in all density fractions (Table 4). The distribution of the two moieties of tritiated cholesterol among density fractions as related to triglyceride hydrolysis is shown in **Fig. 3.** It is apparent that the removal of unesterified cholesterol from VLDL increased with the degree of triglyceride hydrolysis, whereas cholesteryl esters were not removed from the VLDL even when 70–80% of the triglycerides had been hydrolyzed.

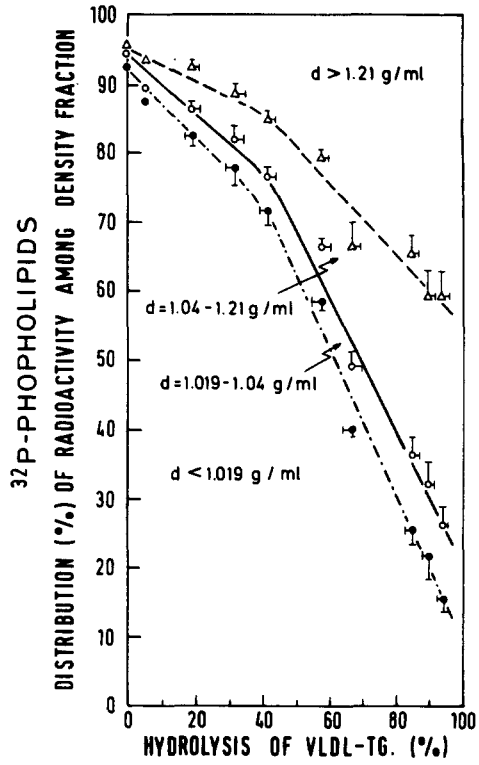

Fig. 1. Distribution (%) of [^{32}P]phospholipids among density fractions during hydrolysis of VLDL triglycerides. Lipolysis was carried out in vitro with lipoprotein lipase purified from bovine milk and rat plasma VLDL labeled with [^{14}C]palmitic acid and [^{32}P]phospholipids, as described in Methods. Following the incubation, fractions were prepared by ultracentrifugation at salt densities of 1.019 g/ml (–·–·–), 1.04 g/ml (○ —— ○), and 1.21 g/ml (△ – – – △). The radioactivity associated with [^{32}P]phospholipids in the four density fractions thus obtained (d < 1.019 g/ml, d 1.019–1.04 g/ml, d 1.04–1.21 g/ml, and d > 1.21 g/ml) was then determined. Values at 0% hydrolysis of VLDL triglycerides (VLDL-TG) represent results of ultracentrifugation of mixtures incubated for 30 min at 37°C without enzyme. Percent hydrolysis of VLDL triglycerides was determined by thin-layer chromatography of aliquots of the incubation mixture. The content of [^{32}P]phospholipids in each density fraction was determined after lipid extraction as described in Methods. Data are based on results obtained from 40 individual experiments, grouped according to the percent hydrolysis of VLDL triglycerides, and are expressed as mean ± SD of hydrolysis of triglycerides and distribution of [^{32}P]phospholipids. VLDL-TG, VLDL triglycerides.

[^{125}I-Labeled apolipoprotein C

The data shown in **Fig. 4** confirm our previous observations (14) and extend them to the whole range of triglyceride hydrolysis. [^{125}I-Labeled apoC was removed from the VLDL and IDL density (d < 1.019 g/ml) throughout the range of triglyceride hydrolysis and, except for the very early and very late points,

[1] Displacement of unesterified cholesterol and apoC during incubation of rat plasma VLDL with albumin solutions was studied in great detail by one of the investigators (S.E.). With the albumin preparation used here (Methods) it was cosistently found that during incubations at 37°C and without lipoprotein lipase, some unesterified cholesterol and apoC were displaced from the VLDL. As shown in Table 3 and Fig. 2, the displaced unesterified cholesterol was found predominantly with the buffer fraction d 1.04–1.21 g/ml; apoC partitioned between the fractions d 1.04–1.21 g/ml and d > 1.21 g/ml, more so in the latter (Fig. 4). This phenomenon was dependent on the ratio of VLDL to albumin, and on the duration and temperature of the incubation. Neither triglycerides nor phospholipids were displaced from the VLDL under identical conditions, at least to any appreciable extent. The displacement of unesterified cholesterol and apoC was remarkably constant and reproducible.

TABLE 2. ^{32}P-Labeled phospholipids in lipoprotein fractions after incubation of ^{32}P-labeled VLDL without and with lipoprotein lipase

Lipolysis %	Density g/ml	[^{32}P]phospholipids, % of Total ^{32}P	^{32}P-Labeled Phospholipids, % of Total ^{32}P				
			OR[a]	LL	SP	LE	PE
[b]	<1.019	92.4 ± 0.5	0.9 ± 0.2	3.7 ± 0.5	6.3 ± 0.5	82.9 ± 0.5	6.2 ± 0.4
35.8 ± 3.5	<1.019	74.3 ± 4.4	1.4 ± 0.4	4.1 ± 0.2	6.8 ± 0.5	84.2 ± 0.3	3.5 ± 0.3
	1.019–1.04	3.3 ± 0.8	0.2 ± 0.1	1.8 ± 0.2	8.7 ± 0.6	87.7 ± 1.3	1.6 ± 0.2
	1.04–1.21	8.6 ± 1.0	1.4 ± 0.7	12.3 ± 3.2	12.1 ± 1.0	70.8 ± 3.9	3.4 ± 0.7
	>1.21	13.8 ± 1.8	0.8 ± 0.3	75.6 ± 1.4	5.3 ± 0.4	16.1 ± 1.0	2.2 ± 0.2
86.5 ± 8.5	<1.019	24.1 ± 2.7	1.4 ± 0.5	4.8 ± 0.5	8.0 ± 0.8	82.4 ± 1.0	3.4 ± 0.6
	1.019–1.04	9.7 ± 0.8	1.8 ± 0.6	4.0 ± 0.7	9.5 ± 0.6	82.4 ± 2.2	2.3 ± 0.2
	1.04–1.21	30.3 ± 2.4	1.7 ± 0.2	8.2 ± 0.2	14.9 ± 1.6	71.7 ± 2.2	3.5 ± 1.2
	>1.21	35.9 ± 4.8	0.4 ± 0.3	74.4 ± 1.5	4.3 ± 1.3	18.7 ± 0.6	2.2 ± 0.4

Procedures of incubation, separation of lipoprotein fractions and of radioactive phospholipids were identical to those described in legend to Table 1. The VLDL used was doubly labeled, with [^{14}C]palmitate and [^{32}P]phospholipids. Lipolysis was determined from the hydrolysis of [^{14}C]palmitate-labeled triglycerides, as described in Table 1. Values are means ± SE of 4–7 experiments.

[a] Abbreviations: OR, origin of plate; LL, lysolecithin; SP, sphingomyelin; LE, lecithin; PE, phosphatidylethanolamine.

[b] Sample incubated at 37°C for 30 min without enzyme.

was linearly related to triglyceride hydrolysis. The ^{125}I-labeled apoC was quantitatively recovered in the density fractions d 1.04–1.21 g/ml and d > 1.21 g/ml. After subtraction of values found in these fractions during incubation without enzyme, the amount of ^{125}I-labeled apoC recovered in the fraction d 1.04–1.21 g/ml was equal, or slightly larger, than that in the fraction d > 1.21 g/ml.

VLDL lipids after incubation without and with lipoprotein lipase

To ascertain that the transfer of labeled lipids from VLDL reflects mass change of the lipoprotein, the content of triglycerides, phospholipids, and total cholesterol in VLDL (d < 1.019 g/ml) was determined at several degrees of lipolysis (**Table 5**). An excellent

TABLE 3. [^{32}P]Phospholipids in the lipoprotein fraction of density 1.04–1.21 g/ml after incubation without and with lipoprotein lipase

Lipoprotein Substrate	Lipoprotein Lipase	^{32}P-Labeled Phospholipids, % of Total ^{32}P		
		LL[a]	SP	LE
(1.04–1.21) g/ml[b]	Absent	4.8 ± 2.4	15.4 ± 1.4	77.2 ± 1.0
(1.04–1.21) g/ml	Present	8.3 ± 1.7	15.7 ± 1.5	74.8 ± 2.6
(1.04–1.21) g/ml + unlabeled VLDL[c]	Present	18.3 ± 1.7	16.6 ± 1.6	62.1 ± 2.4
^{32}P-labeled VLDL	Present	30.5 ± 1.3	6.8 ± 0.9	59.0 ± 3.5

The incubation mixture consisted of aliquots of ^{32}P-labeled phospholipids (1–2 μg of phosphorus) isolated with the fraction of d 1.04–1.21 (see footnote) or of ^{32}P-labeled VLDL (5μg of phosphorus), 0.2 M Tris buffer, pH 8.2, 40 mg of fatty acid-poor bovine albumin, and 5 μl of lipoprotein lipase in a total volume of 1 ml. Incubations were carried out at 37°C for 30 min in a thermostated incubator without shaking and were terminated by the addition of 20 volumes of chloroform–methanol 2:1 (v/v). Phospholipids were separated by thin-layer chromatography after lipid extraction, as described in Methods. Values are mean ± SE of 6 experiments.

[a] Abbreviations: see legend to Table 2.

[b] The fraction d 1.04–1.21 g/ml was obtained from an incubation mixture containing ^{32}P-labeled VLDL, Tris–albumin buffer, fatty acid-poor bovine albumin, and lipoprotein lipase. After the incubation, the fraction d 1.04–1.21 g/ml was isolated by sequential ultracentrifugation as described in Methods and was exhaustively dialyzed against 0.15 M NaCl, 0.001% EDTA, pH = 7.4 buffer. The dialyzed sample was then incubated as described above.

[c] Unlabeled VLDL (5 μg of phosphorus) was mixed with an aliquot of the fraction d 1.04–1.21 g/ml (1–2 μg of phosphorus) and the buffered albumin. The mixture was allowed to equilibrate at 37°C for 30 min prior to the addition of the lipoprotein lipase. Incubations were carried out as described above.

TABLE 4. [^{3}H]Cholesterol in lipoprotein fractions after incubation of ^{3}H-labeled VLDL without and with lipoprotein lipase

Lipolysis %	Density g/ml	[^{3}H]Cholesterol, % of Total ^{3}H	[^{3}H]Cholesterol, % of Total ^{3}H	
			UC[a]	CE
[b]	<1.019	86.4 ± 1.3	70.3 ± 1.9	23.6 ± 1.5
	1.019–1.04	1.4 ± 0.5	ND[c]	ND[c]
	1.04–1.21	9.5 ± 1.1	90.1 ± 1.0	3.4 ± 1.5
31.7 ± 3.0	<1.019	80.0 ± 1.1	67.8 ± 1.2	27.0 ± 1.0
	1.019–1.04	3.7 ± 0.9	62.6 ± 3.8	29.5 ± 4.2
	1.04–1.21	13.4 ± 0.7	88.8 ± 1.4	6.4 ± 1.3
64.9 ± 5.6	<1.019	66.6 ± 2.3	63.3 ± 2.5	31.0 ± 2.1
	1.019–1.04	6.4 ± 1.3	59.5 ± 1.7	26.7 ± 2.7
	1.04–1.21	23.5 ± 1.1	89.1 ± 2.8	9.3 ± 1.1
88.3 ± 4.5	<1.019	30.4 ± 2.2	54.7 ± 3.2	37.2 ± 1.5
	1.019–1.04	14.8 ± 1.3	58.7 ± 3.9	29.3 ± 1.5
	1.04–1.21	49.2 ± 4.4	77.4 ± 1.6	13.5 ± 1.5

Procedures of incubation, separation of lipoproteins and of radioactive cholesterol were identical to those described in legend to Table 1. The VLDL used was doubly labeled, with [^{14}C]palmitate and [^{3}H]cholesterol. Lipolysis was determined from the hydrolysis of [^{14}C]palmitate-labeled triglycerides, as described in Table 1. Values are means ± SE of 3–6 experiments.

[a] Abbreviations: UC, unesterified cholesterol; CE, cholesteryl esters.

[b] Sample incubated at 37°C for 30 min without enzyme.

[c] Not determined.

agreement was found between the radiochemical and chemical determinations.

DISCUSSION

A model for the structure of VLDL, based on compositional, enzymatic, and physical data has been proposed recently by Morrisett, Jackson, and Gotto (11). The particle is described as a sphere with a hydrophobic triglyceride–cholesteryl ester core and a 20 Å wide polar shell composed of proteins, unesterified cholesterol, and phospholipids. The general features of the model are in excellent agreement with the measured amounts of core and surface constituents in VLDL particles of different density, size, and weight (5, 7–9). Hence, triglyceride hydrolysis must be accompanied by a proportional decrease of the surface area of VLDL particles. We have recently demonstrated that during passage of VLDL through the isolated rat heart capillaries, phospholipids, unesterified cholesterol, and apolipoprotein C are removed from the lipoprotein concomitantly with the hydrolysis of triglycerides (13). These experiments were carried out in a perfusion system devoid of plasma and plasma lipoproteins. Therefore, we concluded that the decrease of the VLDL surface area is independent of the presence of an acceptor lipoprotein at the site of lipolysis and reflects the changing chemical and physical properties of the VLDL as induced by lipolysis. The present results confirm this conclusion and extend the previous observations to an in vitro incubation system and an unassociated lipoprotein lipase.

Comparison of the two experiments (in vitro and isolated heart perfusion) reveals many similarities and few differences. Triglyceride hydrolysis was progressive in the two experiments. VLDL and IDL isolated after in vitro lipolysis, however, contained more fatty acids than after the perfusion. Since fatty acids

TABLE 5. Lipid mass in VLDL (d < 1.019) before and during lipolysis

Lipolysis	VLDL Lipids		
	Triglyceride	Phospholipid	Cholesterol
%		*mg*	
0	2.78 ± 0.43	0.36 ± 0.09	0.28 ± 0.04
35.7 ± 3.8	1.81 ± 0.27	0.30 ± 0.04	0.26 ± 0.03
82.3 ± 3.9	0.43 ± 0.06	0.12 ± 0.03	0.15 ± 0.03

Procedure of incubation is identical to that described in legend to Table 1. VLDL and post-lipolysis VLDL were isolated by ultracentrifugation at d < 1.019 g/ml as described in Methods. Lypolysis was determined from the hydrolysis (%) of [^{14}C]palmitate-labeled VLDL triglycerides. Chemical analysis of VLDL and post-lipolysis VLDL was carried out on lipid extracts of the lipoproteins as outlined in the Methods section. The disappearance of radioactive phospholipids and cholesterol from lipolyzed VLDL was measured simultaneously in these samples. At 35.7% lipolysis, 21.6% of the [^{32}P]phospholipids and 8.3% of the [^{3}H]cholesterol have been removed from the VLDL (d < 1.019 g/ml). The corresponding values at 82.3% lipolysis were 72.3% and 64.7%, respectively. Results are mean ± SD of 4–7 experiments.

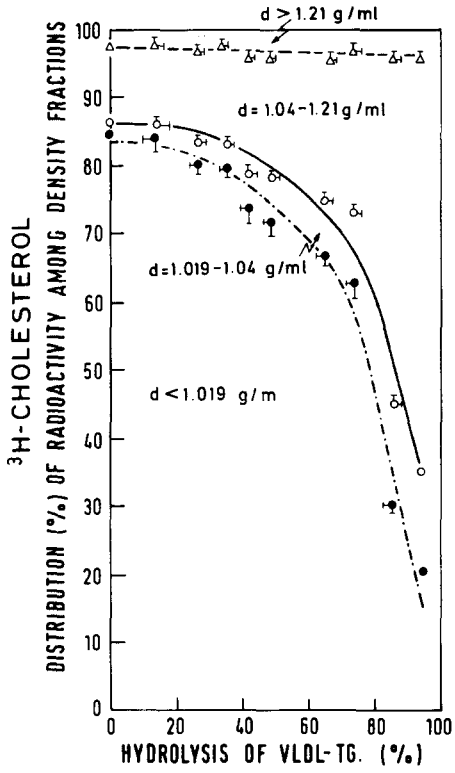

Fig. 2. Distribution (%) of [^{3}H]cholesterol among density fractions during hydrolysis of VLDL triglycerides. Conditions of incubations, determination of hydrolysis of VLDL triglycerides, and distribution of [^{3}H]cholesterol among density fractions were determined as described in legend to Fig. 1. Density fractions were prepared by ultracentrifugation at salt densities of 1.019 g/ml (–·–·–), 1.04 g/ml (○ —— ○), and 1.21 g/ml (△ – – – △). The radioactivity associated with [^{3}H]cholesterol in the four density fractions thus obtained (d < 1.019 g/ml, d 1.019–1.04 g/ml, d 1.04–1.21 g/ml, and d > 1.21 g/ml) was then determined. Values at 0% hydrolysis of VLDL triglycerides represent results of ultracentrifugation of mixtures incubated for 30 min at 37°C without enzyme. Data are based on results obtained in 32 individual experiments grouped according to the percent hydrolysis of VLDL triglycerides and are expressed as mean ± SD of hydrolysis of triglycerides and distribution of [^{3}H]cholesterol. VLDL-TG, VLDL triglycerides.

are avidly taken up by the heart muscle (25) we suggest that lipolysis at the surface of the heart capillaries results in a better extraction of fatty acids than observed with buffered albumin alone. This finding warrants careful characterization of in vitro-produced post-lipolysis VLDL particles, especially when used in experiments where sensitivity to fatty acids may exist, i.e., tissue culture systems. The fate of phospholipids was essentially identical in the two experiments. Pathways of removal of lecithin, generation of lysolecithin, and removal of sphingomyelin were observed in both systems. In both, the observations are consistent with the suggestion that the primary site of hydrolysis of lecithin to lysolecithin is the surface of the VLDL particles. However, more lysolecithin was generated during the in vitro incubation than during the heart perfusion. Whether this difference is due to the different experimental conditions or to the different enzymes is unknown. Yet it is interesting to note that marked phospholipase activity of the milk enzyme has been noted in other studies (26), whereas the heart lipoprotein lipase, even during in vitro incubation with VLDL, did not hydrolyze more than 20–25% of the lipoprotein glycerophosphatides (13).

Conflicting results have been previously reported concerning the fate of unesterified cholesterol in VLDL. Following the injection of heparin to humans (1) or rats (6), the cholesterol content decreases in VLDL and increases in HDL. Decreasing amounts of unesterified cholesterol were found in VLDL particles of decreasing S_f rates (3, 27). Hydrolysis of 80% of the triglycerides in rat plasma VLDL during in vitro incubation with lipoprotein lipase-rich (postheparin) plasma is associated with loss of about one half of the unesterified cholesterol when the lipoprotein particles' weight decreases from 23.1×10^6 daltons to 7.0×10^6 daltons (4, 7). Other investigators did not find appreciable or any loss of unesterified cholesterol during VLDL lipolysis. Mjøs et al. (5) reported identical levels of unesterified cholesterol in rat plasma VLDL (mean particle weight 56.3×10^6 daltons, 73.7% triglycerides) and in VLDL remnants (mean particle weight $24.7–26.6 \times 10^6$ daltons) prepared in the supradiaphragmatic portion of the rat. Fielding and Higgins (28) reported similar results during per-

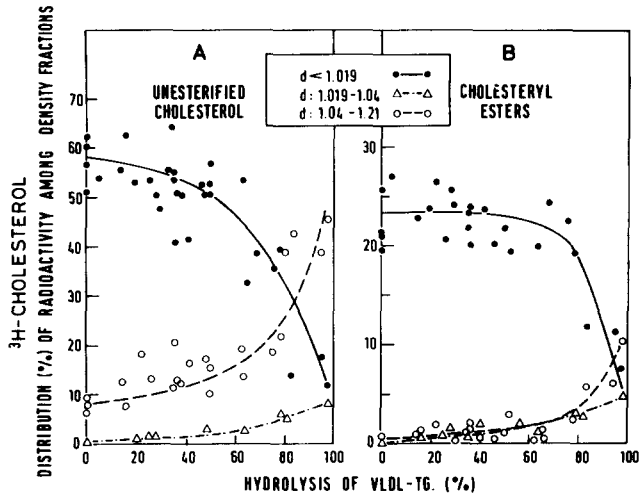

Fig. 3. Distribution (%) of unesterified [^{3}H]cholesterol (A) and ^{3}H-labeled cholesteryl ester (B) among density fractions during hydrolysis of VLDL triglycerides. Data are from the 32 individual experiments described in Fig. 2. After ultracentrifugation, each fraction was isolated, the lipids were extracted in 20 volumes of chloroform–methanol 2:1 (v/v), and unesterified cholesterol and cholesteryl esters were separated by thin-layer chromatography as described in Methods. Data are expressed as percent of total [^{3}H]cholesterol introduced into the incubation mixture. VLDL-TG, VLDL triglycerides.

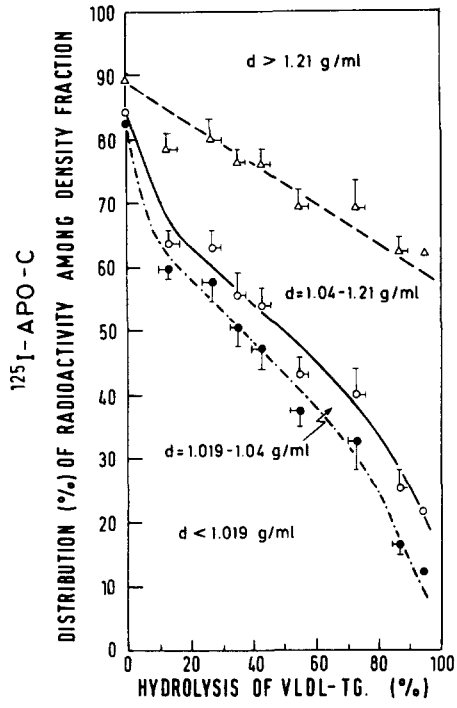

Fig. 4. Distribution (%) of ^{125}I-labeled apoprotein C among density fractions during hydrolysis of VLDL triglycerides. Conditions of incubation, determination of hydrolysis of VLDL triglycerides, and distribution of radioactivity among density fractions were determined as described in legend to Fig. 1. ^{125}I-Labeled apolipoprotein C radioactivity was assessed as described in Methods. Density fractions were prepared by ultracentrifugation at salt densities of 1.019 g/ml (–·–·–), 1.04 g/ml (○ —— ○), and 1.21 g/ml (△ – – – △). The radioactivity associated with ^{125}I-labeled apolipoprotein C in the four density fractions thus obtained (d < 1.019 g/ml, d 1.019–1.04 g/ml, d 1.04–1.21 g/ml, and d > 1.21 g/ml) was then determined. Values at 0% hydrolysis of VLDL triglycerides represent results of ultracentrifugation of mixtures incubated for 30 min at 37°C without enzymes. Data are based on results obtained in 35 individual experiments grouped according to percent hydrolysis of VLDL triglycerides and are expressed as mean ± SD of hydrolysis of triglycerides and distribution of ^{125}I-labeled apolipoprotein C. VLDL-TG, VLDL triglycerides.

fusion of rat plasma VLDL (S_f rate 100–900, 80.7% triglycerides) in the isolated perfused rat heart. In the present investigation we found slow rates of removal of unesterified cholesterol during hydrolysis of the first half of the VLDL triglycerides, but an accelerated rate with further lipolysis (compare 0–50% lipolysis and 50–98% lipolysis, Fig. 3). These changing rates are not adequately explained either by surface to volume considerations or by the depletion of the VLDL of 10–15% of the unesterified cholesterol observed without lipolysis (see Table 4). We suggest that this phenomenon is due, at least in part, to partition of unesterified cholesterol between the triglyceride core and the phospholipid-protein surface monolayer of the VLDL particles as suggested recently for LDL (20) and HDL (30).

A study on the solubility of cholesterol in triolein while using a triolein–water system at 37°C established a 3.2% solubility by weight (7 mol %) of the cholesterol in the triolein (31). The partition of unesterified cholesterol between the core and surface of VLDL is unknown; however, it can be estimated from phase diagrams of unesterified cholesterol, cholesteryl esters, and phospholipids (32). To this end, the partition of unesterified cholesterol between core and surface in several VLDL particles of known weight and composition was calculated. In one example, 33.4% of the unesterified cholesterol molecules of a 56.3×10^6 dalton VLDL (5) are present in the core; only 17.8% are localized at the core of an 8.2×10^6 dalton particle (3), and 82.2% are available at the phospholipid phase. That such distributions actually occur in lipoproteins has been demonstrated for chylomicrons when surface and oil phases have been prepared (33, 34). In dogs' and rats' intact chylomicrons, the unesterified cholesterol to phospholipid weight ratio is 0.155 and 0.060, respectively. This ratio decreased to 0.110 and 0.036 in the separated surface phospholipid-protein fraction, indicating that approximately one third to one half of the unesterified cholesterol was separated with the triglyceride–cholesteryl ester phase. If these calculations are extended to VLDL, it is to be expected that with decreasing size and molecular weight of the particles increasing proportions of unesterified cholesterol may be localized at the phospholipid–apoprotein outer shell. Hence, as the particle becomes smaller a greater fraction of the lipoprotein unesterified cholesterol may be available at the outer shell and will be removed upon further lipolysis and further decrease of the particle's diameter.

Other similarities between the in vitro experiment and the heart perfusion experiment were the progressive removal of apoC and the fate of cholesteryl esters. In the two experiments, apoC was removed to fractions d > 1.21 g/ml and d 1.04–1.21 g/ml, implying that some of the apoC molecules were not lipidated (or associated with small amounts of lipids) whereas other apoC molecules were complexed with lipids. As mentioned above, phospholipids and unesterified cholesterol were also recovered from the fraction d 1.04–1.21 g/ml. The presence of these lipids and apoC at the same density fraction suggested that they may be associated in a lipoprotein form. Indeed, as was previously observed (13, 35), many discoidal lipoproteins were visualized in this fraction by negative-staining electron microscopy.

The appearance of cholesteryl esters in the fraction d 1.019–1.04 g/ml observed at high degrees (>80%) of lipolysis presumably represents the formation of LDL

in vitro, as described recently for human plasma VLDL and similar incubation conditions (35). Cholesteryl esters found with the fraction d 1.04–1.21 g/ml may then represent LDL particles of hydrated density >1.04 g/ml.

It is tempting to speculate that the process of VLDL degradation in vivo is essentially similar to that described here and in the previous report (13). Yet, more studies are obviously needed for a better characterization of the lipolytic products and their relationship to native plasma LDL and HDL particles. The present investigation, and the close resemblance (though not identity) of products formed in vitro and during rat heart perfusion, encourage us to suggest that simple in vitro systems and carefully controlled conditions may be used in the future for the study of the metabolic relationships of lipoproteins.

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