Phospholipid removal during degradation of rat plasma very low density lipoprotein in vitro[1]

Shlomo Eisenberg and Daniel Schurr

Lipid Research Laboratory, Department of Medicine B, Hadassah University Hospital, Jerusalem, Israel

Abstract The hydrolysis of glycerophospholipids in very low density lipoprotein by enzyme(s) released into circulation after the injection of heparin to rats was studied. [^{32}P]Lysolecithin was formed rapidly from [^{32}P]lecithin when very low density lipoprotein, labeled biosynthetically with ^{32}P, was incubated with postheparin plasma. The [^{32}P]lysolecithin was associated with the plasma protein fraction of density greater than 1.21 g/ml, whereas [^{32}P]lecithin exchanged between very low and high density lipoproteins. Inhibition of the plasma lecithin: cholesterol acyl transferase activity did not change the excess [^{32}P]lysolecithin formation in postheparin plasma, and only a negligible amount of radioactivity was associated with blood cells when the incubation was repeated in whole blood.

Analysis of the results has demonstrated that phospholipids are removed from VLDL by two pathways: hydrolysis of glycerophospholipids by the heparin-releasable phospholipase activity (>50%) and transfer to high density lipoproteins (<50%). The tissue origin of the postheparin phospholipase was studied in plasma obtained from intact rats and supradiaphragmatic rats using specific inhibitors of the extrahepatic lipase system (protamine sulfate and 0.5 M NaCl). The phospholipase activity could be ascribed to both the hepatic and extrahepatic lipase systems.

It is concluded that hydrolysis of glycerophospholipids is the major mechanism responsible for the removal of phospholipids from very low density lipoprotein during the degradation of the lipoprotein. It is suggested that phospholipid hydrolysis occurs concomitantly with triglyceride hydrolysis, predominantly in extrahepatic tissues.

Supplementary key words heparin releasable phospholipase activity · lipoprotein-phospholipids · lysolecithin · [^{32}P]lecithin · [^{32}P]sphingomyelin · "hepatic" and "extrahepatic" lipoprotein lipase · supradiaphragmatic rats

Very low density lipoprotein (VLDL) is a complex lipoprotein particle produced in liver and intestine and is involved with the transport of circulating triglycerides of endogenous origin. About 60% of the VLDL mass is triglycerides; cholesterol, phospholipids, and apoproteins each constitute 10–15% of the particle mass (1,2). The catabolism of VLDL is initiated through triglyceride hydrolysis by the enzyme system lipoprotein lipase. Yet, when partially degraded VLDL particles are isolated in vivo (3) or in vitro (4), it can be shown that in comparison to prelipolysis particles they are deficient not only in triglycerides but also in cholesterol and phospholipids as well as several specific apoproteins.

In a recent study we have demonstrated that during the incubation of rat plasma VLDL with lipoprotein lipase-rich plasma obtained from rats injected with heparin, about 60% of the phospholipid-phosphorus disappears from the density range of the VLDL (4). Since postheparin plasma contains a potent phospholipase A activity (5–10), and since lecithin is the major phospholipid of VLDL (1,3), it has been hypothesized that hydrolysis of lecithin may contribute to its removal from the lipoproteins during active lipolysis (7). The present study was undertaken to test this hypothesis using VLDL labeled biosynthetically with ^{32}P-labeled phospholipids and an in vitro incubation system.

MATERIALS AND METHODS

Preparation of ^{32}P-labeled and [^{3}H]cholesterol-labeled very low density lipoprotein

Male rats (200 g body wt) of the Hebrew University strain, while under ether anesthesia, were injected intravenously with 3–5 mCi of H_3 $^{32}PO_4$ through an exposed saphenous vein. The rats were exsanguinated 16 hr after the injection through the abdominal aorta and blood was collected in 0.1% ethylenediaminotetraacetate (EDTA) solution, pH 7.45. Plasma was separated in a Sorval SS-3

Abbreviations: VLDL, very low density lipoproteins; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyl transferase; PCMPS, *p*-chloromercuriphenylsulfonic acid.

[1] Presented in part at the 1975 meeting of the American Heart Association (*Circulation*. **51–52,** Supp. II. 17).

centrifuge (Ivan Sorval, Norwalk, Conn.) at 4°C and 15,000 rpm for 15 min. VLDL was isolated at plasma density (d 1.006 g/ml) using the L2-65B or L3-50 Beckman ultracentrifuge (Spinco Div., Palo Alto, Cal.) and the 40.3 rotor at 40,000 rpm for 16–18 hr. About 10% of labeled phospholipids in plasma was associated with VLDL. VLDL labeled biosynthetically with [^{3}H]cholesterol was prepared similarly from rats injected intravenously with 10–50 μCi of tritiated cholesterol.

Isolation of lipoproteins

Lipoproteins of d < 1.019 g/ml (predominantly VLDL), and d 1.019–1.04 and 1.04–1.21 g/ml, and the plasma protein fraction of d > 1.21 g/ml were isolated sequentially by preparative ultracentrifugation following the procedure of Havel, Eder, and Bragdon (11). Density was adjusted with solid KBr or KBr solution of known density, and ultracentrifugation was carried out in the Beckman L2-65B or L3-50 ultracentrifuge and the 40.3 rotor at 40,000 rpm for 18 hr (d < 1.019 and d 1.019–1.04 g/ml) or 48 hr (d > 1.21 g/ml). Plasma lipid and lipoprotein levels were similar to those reported previously for rats of the Hebrew University strain (12, 13). Lipoproteins were dialysed against 3–4 changes of 2 l of 0.9% NaCl, 0.01% EDTA solution, pH 7.45. All procedures were carried out at 4°C.

Preparation of postheparin plasma

Postheparin plasma from intact rats was obtained 10 min after the intravenous injection of 100 units/l kg body wt of sodium heparin (Pularin, Evans Medical Ltd., Liverpool, England) dissolved in 0.9% NaCl solution. Normal (control) rat plasma was collected in heparin from noninjected rats. To prepare postheparin plasma free of enzymes released by the liver, supradiaphragmatic rats were prepared by the method of Bezman-Tarcher and Robinson (14). A polyethylene cannula was introduced into the inferior vena cava of ether-anesthetized male rats (200 g body wt) and pushed into position above the diaphragm. The inferior vena cava was then ligated above the origin of the hepatic veins and below the diaphragm, and the aorta was clamped by a hemostat below the diaphragm and above the origin of the celiac trunk. Sodium heparin (100 units/kg) was injected into the supradiaphragmatic portion of the rats through the cannula in the inferior vena cava and blood was collected from the aorta 5 min after the injection of heparin.

Plasma was separated in the Sorval SS-3 centrifuge at 4°C and 15,000 rpm for 15 min. The plasma was used within 60 min of bleeding.

Analytical procedures

Lipids were extracted with chloroform–methanol 2:1 (v/v) and washed as described by Folch, Lees, and Sloane Stanley (15). Phospholipids were separated on HR silica gel thin-layer chromatography plates developed in chloroform–methanol–acetic acid–water 100:50:18:8. Phospholipids were visualized by iodine vapor and identified with the help of reference standards. Phospholipid phosphorus was determined by the method of Bartlett (16). Free and esterified cholesterol were separated by thin-layer chromatography on silica gel G plates developed in petroleum ether–diethyl ether–acetic acid 90:10:1. Cholesterol was determined as described by Chiamori and Henri (17). Triglycerides were determined by the Autoanalyzer method (18). Radioactivity was determined in a Packard model 544 β-liquid scintillation spectrometer. $H_3{}^{32}PO_4$, carrier free, was purchased from the Nuclear Research Centre, Beer-Sheva, Israel, and [^{3}H]cholesterol from the Radiochemical Centre, Amersham, England.

Experimental procedures

Incubation mixtures consisted of ^{32}P-labeled VLDL, normal or postheparin rat plasma, and fatty acid-poor bovine albumin (Pentex, Miles Laboratories, Kankakee, Ill.). The final concentration of plasma was between 60 and 80% (v/v) and that of the albumin between 2 and 4% (w/v), according to the experiments performed (see legend to tables). In most experiments the final volume was 5 ml and the incubation was carried out in 6.5-ml cellulose nitrate ultracentrifuge tubes. The incubation mixture contained 2–2.5 mg of VLDL triglycerides (12), 0.4–0.5 mg of VLDL phospholipids (12), 150 mg of albumin present in the rat plasma, and 100 mg of the added fatty acid-poor bovine albumin (less than 0.1 μmole of free fatty acid/μmole of albumin). The incubation was terminated by the addition of concentrated NaCl solution (d 1.1168 g/ml) to the mixture in a quantity sufficient to bring the density of the mixture to 1.019 g/ml (0.47 M NaCl), and lipoproteins were separated by ultracentrifugation as described above. Using these conditions, only small changes were observed in the distribution of radioactivity among phospholipid classes at time zero (see Table 1). When the experiments were carried out without separation of lipoproteins (Tables 5–7), the incubation volume was 1 ml, and it contained 0.6–0.8 ml of plasma (0.4–0.5 mg triglycerides) and fatty acid-poor bovine albumin at a final concentration

TABLE 1. Distribution of radioactivity among phospholipids during incubation[a] of ^{32}P-labeled VLDL with normal or postheparin plasma

Source of Plasma	Time of Incubation	Distribution of ^{32}P-labeled Phospholipids				
		Origin	LL	SP	PC	PI + PE
	min			%[b]		
VLDL, original		0.3 ± 0.2	1.2 ± 0.4	5.6 ± 0.5	88.6 ± 0.6	4.3 ± 0.4
Normal	60	0.5 ± 0.2	7.7 ± 1.0	9.4 ± 0.8	78.8 ± 0.8	3.5 ± 0.3
Postheparin	0	0.5 ± 0.2	5.7 ± 0.5	7.9 ± 0.3	82.8 ± 1.0	3.1 ± 0.4
	5	0.2 ± 0.1	10.7 ± 0.7	8.5 ± 0.9	79.9 ± 0.3	1.6 ± 0.5
	15	0.6 ± 0.4	14.3 ± 0.5	8.5 ± 0.2	75.2 ± 1.0	1.4 ± 0.2
	30	0.3 ± 0.2	19.6 ± 0.8	9.5 ± 1.0	69.2 ± 1.6	1.3 ± 0.3
	60	0.4 ± 0.2	29.3 ± 1.1	8.6 ± 0.7	60.0 ± 1.5	2.1 ± 0.5
Postheparin heated[c]	60	0.4 ± 0.2	6.4 ± 0.4	7.0 ± 0.4	82.7 ± 0.4	3.6 ± 0.2

[a] ^{32}P-Labeled VLDL, 0.1–0.3 ml, (1–2 μg of phospholipid-phosphorus, (3–10) × 10^3 cpm) was incubated at 37°C with 4 ml of plasma obtained from either normal rats or rats injected with heparin, and with 0.5 ml of 20% fatty acid-poor bovine albumin. Final volume was 5 ml. The distribution of ^{32}P-labeled phospholipids in each sample was determined by thin-layer chromatography as described in Methods.

[b] Values are means ± SE of six experiments.

[c] Sample heated at 56°C for 30 min prior to incubation.

LL, Lysolecithin; SP, sphingomyelin; PC, phosphatidylcholine (lecithin); PI + PE, a combined fraction of phosphatidylinositol and phosphatidylethanolamine.

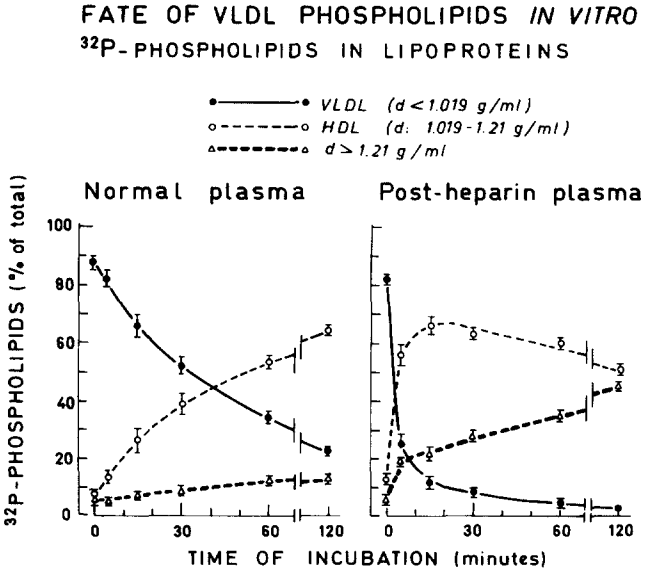

Fig. 1. ^{32}P-Labeled VLDL was incubated in 6.5-ml cellulose nitrate ultracentrifuge tubes with either normal or postheparin plasma as described in legend to Table 1. At the end of the incubation, the tubes were chilled in crushed ice, and lipoproteins were separated by ultracentrifugation in a Beckman ultracentrifuge and the 40.3 rotor as described in Methods. Radioactivity in lipoproteins was determined after lipid extraction. VLDL was isolated at the d < 1.019 g/ml and HDL at the density interval of 1.019–1.21 g/ml. The higher density lipoproteins were separated at two densities 1.019–1.04 g/ml and 1.04–1.21 g/ml, in three of the four experiments. However, since the d 1.019–1.04 g/ml fraction contained 2–4% of total radioactivity, and phospholipid-P in all samples, it was subsequently combined with the major fraction of d 1.04–1.21 g/ml. Results are mean ± SE of four experiments.

of 4% (40 mg of albumin). The incubations were terminated by the addition of 20 ml of chloroform–methanol 2:1 (v/v). Following lipid extraction, the recovery of ^{32}P-labeled phospholipids in the chloroform phase was complete as compared to that of nonincubated ^{32}P-labeled VLDL.

RESULTS

VLDL labeled biosynthetically with ^{32}P-labeled phospholipids contained 1.2% of the radioactivity associated with lysolecithin, 5.6% with sphingomyelin, 88.6% with phosphatidylcholine, and 4.3% with a combined fraction of phosphatidylinositol and phosphatidylethanolamine (**Table 1**). Incubation of ^{32}P-labeled VLDL with normal plasma for 60 min resulted in an increase of [^{32}P]lysolecithin to 7.7% of total radioactivity, and a decrease of [^{32}P]lecithin to 78.8% (Table 1). During incubation of the ^{32}P-labeled VLDL with postheparin plasma, generation of [^{32}P]lysolecithin was much more pronounced, and at the end of 60 min it accounted for 29.3% of total radioactivity. There was also a definite decrease of the small amount of radioactivity associated with the combined fraction of phosphatidylinositol and phosphatidylethanolamine to 1.3–2.1% of the radioactivity (Table 1). These changes were abolished when the postheparin plasma was heated for 30 min at 56°C (Table 1).

TABLE 2. ^{32}P-Labeled phospholipids of lipoproteins during incubation of ^{32}P-labeled VLDL with normal or postheparin plasma

Lipoprotein	Source of Plasma	Incubation Time	Distribution of ^{32}P-Labeled Phosphilipids				
			Origin	LL	SP	PC	PI + PE
		min			*% of total*[a]		
d < 1.019	Normal	0	0.4 ± 0.1	2.6 ± 0.3	7.0 ± 0.6	85.8 ± 1.0	4.2 ± 0.4
		60	0.9 ± 0.3	3.3 ± 0.5	9.5 ± 0.9	80.7 ± 1.2	5.6 ± 0.7
	Postheparin	0	1.2 ± 0.9	2.7 ± 0.4	8.3 ± 1.7	83.5 ± 2.9	4.3 ± 0.7
		5	0.4 ± 0.2	1.3 ± 1.0	11.2 ± 2.1	82.9 ± 0.6	4.2 ± 1.1
		15	0.5 ± 0.3	3.0 ± 1.1	16.8 ± 2.9	77.0 ± 1.2	2.9 ± 0.8
		30	0.6 ± 0.2	4.2 ± 2.0	19.0 ± 2.9	73.0 ± 2.7	3.2 ± 1.0
		60	1.3 ± 0.2	3.5 ± 0.5	24.1 ± 2.7	64.7 ± 2.9	6.4 ± 1.1
	Postheparin, heated	60	0.3 ± 0.2	3.3 ± 0.6	7.4 ± 1.0	84.4 ± 1.9	4.7 ± 0.3
d 1.019–1.21[b]	Normal	60	0.8 ± 0.2	6.6 ± 1.1	7.4 ± 1.8	82.5 ± 2.6	2.6 ± 0.3
	Postheparin	60	0.6 ± 0.2	7.9 ± 0.7	10.1 ± 0.8	80.2 ± 0.8	1.2 ± 0.3
d > 1.21[b]	Normal	60	0.7 ± 0.1	81.5 ± 2.3	3.4 ± 0.5	13.4 ± 1.8	1.0 ± 0.6
	Postheparin	60	3.4 ± 1.3	88.8 ± 1.7	2.5 ± 0.9	4.9 ± 1.4	0.4 ± 0.1

[a] Mean ± SE of four experiments. Lipoproteins were isolated during incubations as described in Table 1. The distribution of radioactivity among phospholipid was determined by thin-layer chromatography as described in Methods.

[b] The distribution of radioactivity among phospholipids in lipoproteins was determined at all time intervals. Except for samples of very low density lipoprotein (d < 1.019 g/ml), the results at all time intervals were similar to those shown in the table.

Transfer of ^{32}P-labeled phospholipids from VLDL to higher density lipoproteins (d 1.019–1.21 g/ml) and to the plasma protein fraction of d > 1.21 g/ml was recorded during the incubation with both normal and post-heparin plasma (**Fig. 1**). The transfer of ^{32}P-labeled phospholipids to higher density lipoproteins and their appearance in the plasma fraction of d > 1.21 g/ml were much more rapid with post-heparin plasma, especially during the first 5–15 min of the incubation. As shown below (**Table 2**), the predominant phospholipid of the high density lipoproteins was [^{32}P]lecithin and that of the plasma fraction of d > 1.21 g/ml was [^{32}P]lysolecithin.

Phospholipid specific activity curves were determined throughout the experiment using the isolated lipoprotein fractions (**Fig. 2**). The specific activity of phospholipids in VLDL and HDL approached one another at the end of 60–120 min, and it was of similar magnitude in the two plasma samples. The decrease of phospholipid specific activity in VLDL and its increase in HDL during the first 15 min, however, was much more pronounced in samples incubated with postheparin plasma as compared to normal plasma. During this time interval we also observed the steepest rise in ^{32}P-labeled phospholipid in HDL and the plasma fraction of d > 1.21 g/ml as well as [^{32}P]lysolecithin generation. We therefore suggest that the pronounced changes of specific activity of VLDL and HDL ^{32}P-labeled

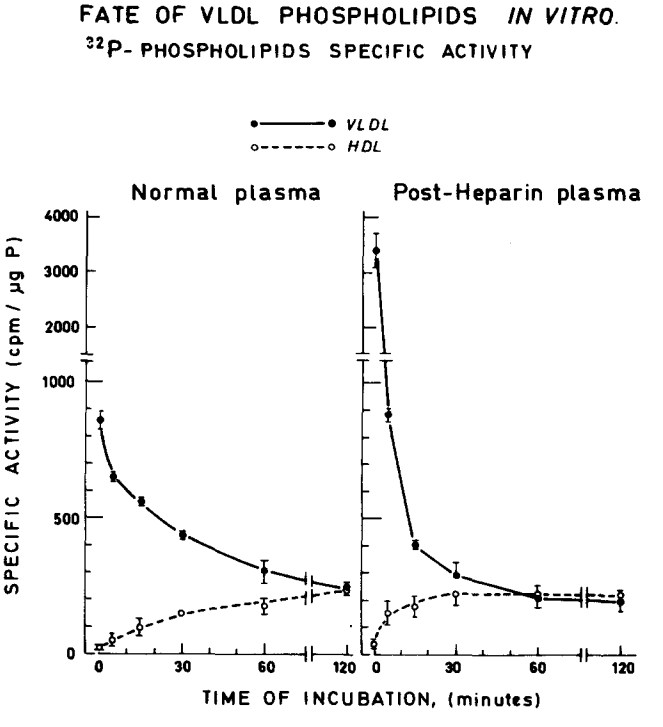

Fig. 2. Specific activity of ^{32}P-labeled phospholipids in very low (d < 1.019 g/ml) and high (d 1.019–1.21 g/ml) density lipoproteins during in vitro incubation of ^{32}P-labeled VLDL with normal plasma and plasma obtained from rats injected with heparin. Lipoproteins were isolated during incubations described in legend to Fig. 1. Specific activities were determined after lipid extraction and assay of the extracts for radioactivity and phospholipid-P content.

TABLE 3. Change of lipoprotein-lipids during incubation of normal and postheparin plasma in vitro[a]

Incubation Time	Phospholipid-P				Cholesterol			
	d < 1.019	d 1.019–1.063	d 1.063–1.21	d > 1.21	d < 1.019	d 1.019–1.063	d 1.063–1.21	d > 1.21
min	*% of total*[b]				*% of total*[b]			
0	11.5 ± 0.3	11.8 ± 0.9	47.2 ± 0.7	29.4 ± 1.0	11.8 ± 1.2	23.1 ± 1.9	59.1 ± 1.3	6.0 ± 0.2
5	9.6 ± 0.3	12.7 ± 1.3	47.3 ± 0.6	30.3 ± 0.8	11.1 ± 1.2	22.7 ± 1.7	59.6 ± 0.6	6.6 ± 1.2
15	7.4 ± 0.2	12.3 ± 0.9	47.3 ± 0.4	33.0 ± 0.6	9.9 ± 0.5	22.7 ± 1.3	62.2 ± 1.6	5.2 ± 0.2
30	6.1 ± 0.5	12.7 ± 1.1	47.5 ± 0.5	33.7 ± 1.3	8.3 ± 1.1	22.0 ± 1.8	63.1 ± 6.8	6.5 ± 0.7

[a] Normal rat plasma (8 ml) was incubated at 37°C with 4 ml plasma obtained from rats injected with heparin 10 min prior to exsanguination. Lipoprotein lipids were determined after lipoprotein isolation as described in Methods. Plasma phospholipid levels of the four groups at the end of incubation were 66.1 ± 3.9; 63.8 ± 3.8; 66.0 ± 2.9; and 70.0 ± 4.1 mg/dl, respectively, and cholesterol was 55.6 ± 1.7; 53.8 ± 0.6; 61.9 ± 0.5; and 60.9 ± 1.6 mg/dl, respectively.
[b] Means ± SE of three experiments.

phospholipid are related to the rapid decrease of phospholipid content of VLDL occurring during the first 15 min of incubation, as well as to net transfer of ^{32}P-labeled phospholipids from VLDL to HDL (see Discussion).

The distribution of ^{32}P among phospholipids was determined in all lipoproteins isolated during the in vitro incubation. [^{32}P]Lecithin constituted more than 80% of labeled phospholipids of lipoproteins of density 1.019–1.21 g/ml and [^{32}P]lysolecithin more than 80% of the ^{32}P-labeled phospholipid of the protein fraction of d > 1.21 g/ml (Table 2). In all samples the ^{32}P-labeled phospholipid composition of VLDL (d < 1.019 g/ml) changed during the incubation, especially in samples incubated with postheparin plasma, which became rich in [^{32}P]sphingomyelin and poor in [^{32}P]lecithin. This change diminished when ^{32}P-labeled VLDL was incubated with heat-inactivated postheparin plasma.

The disappearance of phospholipids from VLDL was next measured in an incubation mixture consisting of 8 ml of normal rat plasma and 4 ml of postheparin rat plasma (**Table 3**). During the incubation, about 45–50% of the phospholipids present originally in VLDL were removed from the density range of VLDL, and were found in association with the plasma proteins of d > 1.21 g/ml. In agreement with previous reports (19), this fraction contained predominantly (≥80%) lysolecithin. Cholesterol also disappeared from the VLDL density range, but it was found in HDL (Table 3). Phospholipid analysis of the mixed plasma samples showed an increase in lysolecithin from 20.3 to 28.4% of total phospholipid P (**Table 4**).

Two different enzyme systems present in postheparin plasma may hydrolyze a fatty acid from glycerophospholipids and generate lysoglycerophospholipids: the heparin-releasable phospholipase and the lecithin: cholesterol acyl transferase (LCAT). In order to differentiate between the activities of the two enzymes, we measured [^{32}P]lysolecithin formation in normal and postheparin plasma in the absence and presence of *P*-chloromercuriphenylsulfonic acid (PCMPS, Sigma Chemical Co., St. Louis, Mo.) a known inhibitor of the LCAT system (20). Cholesterol esterification was measured in parallel using VLDL labeled biosynthetically with tritiated cholesterol (**Table 5**). PCMPS completely inhibited [^{32}P]lysolecithin generation and cholesterol esterification in normal plasma, as well as cholesterol esterification in postheparin plasma. [^{32}P]Lysolecithin generation in postheparin plasma changed only slightly,

TABLE 4. Plasma phospholipid composition during incubation of normal and postheparin plasma

Incubation Time	Distribution of Phospholipid-P				
	Origin	LL	SP	PC	PI + PE
min	*% of total*[a]				
0	2.2 ± 0.8	20.3 ± 0.7	5.3 ± 0.3	67.9 ± 0.9	4.8 ± 0.1
5	1.8 ± 0.4	23.2 ± 0.9	4.8 ± 1.3	66.1 ± 2.0	3.8 ± 0.4
15	1.8 ± 1.0	25.5 ± 1.3	5.3 ± 1.6	62.9 ± 2.1	4.3 ± 0.6
30	2.3 ± 0.3	28.4 ± 3.2	6.0 ± 1.5	59.6 ± 0.6	3.7 ± 0.7

[a] Means ± SE of three experiments. Plasma samples were obtained during the experiments described in Table 3. Phospholipid distribution was determined by thin-layer chromatography as described in Methods.

TABLE 5. Effect of inhibition of the lecithin-cholesterol acyl transferase (LCAT) on [^{32}P]lysolecithin formation and cholesterol esterification in normal and postheparin plasma

Source of Plasma	Additives	[^{32}P]Lysolecithin			[^{3}H]Cholesterol Free/Ester Ratio	
		0 min	15 min	60 min	0 min	60 min
		% of total ^{32}P-labeled phospholipids				
Normal		4.0 ± 0.4	4.9 ± 1.1	11.2 ± 0.1	2.32 ± 0.11	1.46 ± 0.05
	PCMPS			4.2 ± 0.2		2.61 ± 0.23
Postheparin		5.5 ± 0.3	16.9 ± 1.0	38.8 ± 1.1	2.50 ± 0.15	1.66 ± 0.05
	PCMPS		13.5 ± 0.3	29.1 ± 0.9		2.92 ± 0.24

VLDL labeled with ^{32}P-labeled phospholipids or [^{3}H]cholesterol, 0.1 ml, was incubated with 0.6 ml of plasma and 0.2 ml of 20% fatty acid-poor bovine albumin at 37°C in a thermostated bath. PCMPS, final concentration of 2 mM, was added to samples as indicated in the table. Incubations were terminated by the addition of 20 ml of chloroform–methanol 2:1 (v/v), and [^{32}P]lysolecithin formation or [^{3}H]cholesterol esterification was determined by thin-layer chromatography as described in Methods.

from 38.8% to 29.1%. PCMPS therefore suppressed [^{32}P]lysolecithin formation similarly in the two systems, with a 7.0% decrease in normal plasma, and a 9.7% decrease in postheparin plasma. The excess [^{32}P]lysolecithin formation observed with postheparin plasma, however, was unaffected by PCMPS. We have therefore concluded that the heparin-releasable phospholipase activity was the major pathway of lecithin hydrolysis in postheparin plasma.

Since postheparin plasma contains at least two species of lipases, of hepatic and extrahepatic origin (see Discussion), we attempted to determine whether the postheparin phospholipase activity is associated with one or both enzyme systems. To this end, the phospholipase activity of postheparin plasma obtained from intact rats (containing both enzymes) was compared to that of postheparin plasma obtained from supradiaphragmatic rats (containing the extrahepatic enzyme only). The postheparin plasma obtained from supradiaphragmatic rats contained considerable phospholipase activity (**Table 6**). Preincubation of postheparin plasma obtained from intact rats with either protamine sulphate (3 mg/ml) or 0.5 M NaCl resulted in an average decrease of [^{32}P]lysolecithin formation of 20–40%. The inhibitory effects of preincubation with protamine sulfate and 0.5 M NaCl were much more pronounced in plasma samples obtained from supradiaphragmatic rats.

The previous experiments were carried out in separated plasma, without a potential cell acceptor for phospholipids. We therefore repeated the in vitro incubation experiment using whole blood rather than plasma (**Table 7**). During 60 min of incubation, only small amounts of the ^{32}P-labeled phospholipids, added to whole blood as ^{32}P-labeled VLDL, were found in association with cells. [^{32}P]Lysolecithin generation, however, proceeded at a rate similar to that observed in the other experiments, and it was associated with the separated plasma fraction.

DISCUSSION

Phospholipids are an integral lipid constituent of all plasma lipoproteins and constitute 8–40% of the lipid mass of various lipoproteins. Phosphatidylcholine, the predominant phospholipid of the lipoprotein system, accounts for more than 80% of total phospholipids of chylomicrons, VLDL and HDL,

TABLE 6. Comparison of phospholipase activity of postheparin plasma obtained from intact and supradiaphragmatic rats

Preincubation	Time of Incubation	[^{32}P]Lysolecithin	
		Intact Rats	Supradiaphragmatic Rats
	min	*% of total radioactivity*	
	0	5.6 ± 0.2	3.7 ± 0.4
	15	13.8 ± 0.1	12.8 ± 1.0
	60	28.7 ± 1.2	25.1 ± 2.4
Protamine sulfate	15	11.6 ± 0.3	4.1 ± 0.3
	60	20.1 ± 0.7	12.0 ± 0.1
0.5 M NaCl	15	9.5 ± 1.7	5.8 ± 0.2
	60	18.7 ± 0.6	12.1 ± 0.1

Mean ± SE of three experiments. Postheparin plasma was obtained from either intact or supradiaphragmatic rats injected intravenously with sodium heparin (100 units/kg) 5 min prior to exsanguination. The incubation mixture consisted of 0.8 ml of plasma, 0.2 ml of 20% fatty acid-poor bovine albumin, and 0.05 ml of ^{32}P-labeled VLDL (0.5–1.5 μg P, 3000–5000 cpm). Preincubation of the plasma with protamine sulfate (3 mg/ml) or NaCl (0.5 M, final concentration) was carried out at 27°C for 30 min. Incubations were carried out at 37°C and were terminated by the addition of 20 ml of chloroform–methanol 2:1 (v/v). Distribution of radioactivity among phospholipids was determined by thin-layer chromatography as described in Methods.

TABLE 7. Partition of ^{32}P-labeled phospholipids between blood cells and plasma during incubation of ^{32}P-labeled VLDL with whole blood obtained from normal and heparin-injected rats

Source of Plasma	Incubation Time	^{32}P-Labeled Phospholipids		^{32}P-Labeled Phospholipids in Plasma				
		Cells	Plasma	Origin	LL	SP	PC	PI + PE
	min	*% of total*		*% of total*				
Normal	0	3.4 ± 0.7	96.6 ± 1.5	1.3 ± 0.1	4.8 ± 0.3	5.7 ± 0.1	85.4 ± 0.1	2.8 ± 0.3
	15	5.5 ± 0.3	94.5 ± 0.4	1.4 ± 0.2	5.9 ± 0.1	6.2 ± 0.2	83.5 ± 0.5	3.0 ± 0.1
	60	7.0 ± 1.0	93.0 ± 1.0	1.0 ± 0.2	7.9 ± 0.4	6.0 ± 0.6	82.4 ± 1.5	2.7 ± 0.3
Postheparin	0	2.6 ± 0.5	97.4 ± 0.5	1.1 ± 0.3	5.2 ± 0.1	6.3 ± 0.5	84.2 ± 0.2	3.0 ± 0.1
	15	7.2 ± 0.7	92.8 ± 1.1	1.4 ± 0.3	28.4 ± 1.1	8.1 ± 0.7	61.2 ± 1.3	0.8 ± 0.2
	60	8.7 ± 0.7	91.3 ± 0.7	1.2 ± 0.1	44.3 ± 1.2	8.1 ± 0.4	45.1 ± 1.1	1.0 ± 0.4

Mean ± SE of three experiments.

Whole blood was obtained in heparin (20 units/ml) from uninjected rats, or 10 min after the intravenous injection of heparin (100 units/kg body wt). The incubation system consisted of 0.8 ml of blood, 0.8 ml of 8% fatty acid-poor bovine albumin, and 0.1 ml of ^{32}P-labeled VLDL (1–2 μg P, (4–6) × 10^3 cpm). Incubations were carried out at 37°C, and terminated by transfer of tubes to crushed ice. Cells and plasma were separated immediately in a refrigerated (4°C) centrifuge at 3000 rpm for 10 min, and the cells were washed twice in ice cold 0.9% NaCl. Lipid extraction and distribution of radioactivity among phospholipids was determined as described in Methods.

and about 60% of the LDL phospholipids (1). It is generally assumed that phospholipids (together with unesterified cholesterol and proteins) are present at the surface of lipoproteins whereas triglycerides and cholesteryl esters occupy their core (21). Therefore, because of simple surface to volume considerations, removal of core constituents—as exemplified during triglyceride hydrolysis—should be accompanied by removal or rearrangement of surface constituents. Several studies have indeed shown that during the degradation of VLDL either in vivo (2, 7), in vitro (4) or in isolated organs (22, 23), more than one-half of the phospholipids present in the original particles are removed, as are several specific apoproteins and unesterified cholesterol. The present investigation was undertaken to study the mechanism of removal of phospholipids from VLDL during in vitro incubation with lipoprotein lipase-rich (postheparin) plasma.

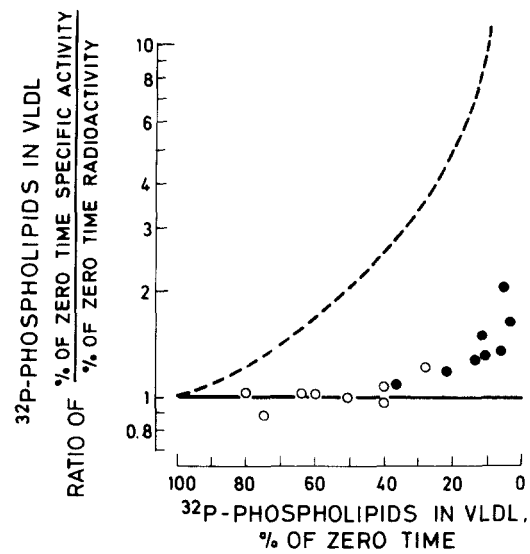

Fig. 3. The relationship between the amount of radioactivity remaining in VLDL (% of zero time, abscissa) and the ratio of % specific activity to % radioactivity remaining in VLDL (ordinate). Solid and broken lines represent theoretical relations of exchange only and transfer only of ^{32}P-labeled phospholipids from VLDL (see Appendix). Open and closed circles represent experimental data, obtained throughout the experiment described in Figs. 1 and 2, during incubations of ^{32}P-labeled VLDL with normal and postheparin plasma, respectively.

Three possible mechanisms were considered for the process of removal of phospholipids from VLDL particles: transfer to other lipoproteins and blood cells; hydrolysis by the heparin-releasable phospholipase activity; and the action of the lecithin: cholesterol acyl transferase (LCAT) enzyme system.

Two main differences were found between postheparin plasma and normal plasma: (*a*) a rapid transfer of ^{32}P-labeled phospholipids from VLDL to HDL, and (*b*) generation of [^{32}P]lysolecithin. Both pathways may explain the removal of glycerophospholipids during degradation of VLDL by lipoprotein lipase-rich (postheparin) plasma. However, since phospholipids do exchange between lipoproteins (as documented in the present report by the decrease in VLDL ^{32}P-labeled phospholipid specific activity, Fig. 2), we have attempted to assess the relative contributions of exchange and of net transfer of ^{32}P-labeled phospholipids from VLDL to HDL.

This assessment was done by calculating the ratio of the phospholipid specific activity to that of ^{32}P-labeled phospholipid radioactivity in VLDL. First, we have drawn two theoretical curves; curve one (solid line, **Fig. 3**) represents exchange only and shows that the ratio of ^{32}P-labeled phospholipid specific activity to that of ^{32}P-labeled phospholipid radioactivity remaining in VLDL is one, regardless of

the amount of radioactive phospholipid removed from VLDL (see Appendix); the second curve (broken line, Fig. 3) represents transfer only and shows that this ratio constantly increases during the removal of the radioactive phospholipids (see Appendix). Second, we have plotted the experimental data (as obtained during the experiments described in Figs. 1 and 2) for incubation of ^{32}P-labeled VLDL with normal plasma (open symbols, Fig. 3) and postheparin plasma (closed symbols, Fig. 3). As expected, the observed values of the ratio of ^{32}P-labeled phospholipid specific activity to that of ^{32}P-labeled phospholipid radioactivity remaining in VLDL during incubation with normal plasma did not deviate much from one. Therefore, the transfer of ^{32}P-labeled phospholipid from VLDL to HDL in this experiment must represent a pure exchange reaction.

A deviation from unity, however, was found during incubation of ^{32}P-labeled VLDL with postheparin plasma. This was expected, since some of the VLDL phospholipids were hydrolyzed to lyso-compounds (see below) and were removed to the plasma protein fraction of density >1.21 g/ml. Thus, at the end of a 5-min incubation, when 65–80% of the ^{32}P-labeled phospholipids disappeared from VLDL (range of individual values used in Fig. 1) the ratio was 1.08–1.19 (see Fig. 3). These values indicate that 7–16% of the labeled phospholipids disappeared from VLDL by a net transfer process, and the other 84–93% by exchange with unlabeled HDL phospholipids. Since the increase of [^{32}P]lysolecithin during these 5 min was 6–12% (range of individual values used in Fig. 1) no more than one-fourth to one-third of the VLDL phospholipids could have been transferred to HDL. Similar calculations show a net removal of ^{32}P-labeled phospholipids of 23% at the end of 15 min (ratio of 1.30) together with a 13% increase of lysolecithin, or a maximal net transfer to HDL of 40% of the VLDL phospholipids. At 30 and 60 min, with ratios of 1.42 and 1.87, respectively, the corresponding values for net transfer of ^{32}P-labeled phospholipids from VLDL were 30% and 46%, [^{32}P]lysolecithin formation was 18% and 24%, and the maximal net transfer to HDL was 40% and 48%, respectively.

Thus, throughout the experiment, glycerophospholipids were removed from VLDL by two pathways: transfer to HDL (25–50% of total phospholipids removed) and hydrolysis to lyso-compounds (50–75% of total phospholipids removed). Since lysolecithin formation was not suppressed when the LCAT system was inhibited (Table 5) and HDL is a poor substrate for the lipoprotein lipase[2], we conclude that the hydrolysis of lecithin occurred through the activity of the plasma heparin releasable phospholipase, and that VLDL was the lipoprotein substrate of the reaction.

The release of a potent phospholipase into the circulation by the injection of heparin was first reported by Vogel and Zieve (5). The release of the enzyme occurs concomitantly with other lipolytic activities (triglyceride and monoglyceride hydrolases) and its activity towards phospholipids, especially an artificial emulsion of phosphatidylethanolamine, has been studied extensively (5–10). The present study extends these previous observations by using a natural lipoprotein substrate, VLDL, labeled biosynthetically with ^{32}P, with postheparin plasma as the enzyme source. In this system the main hydrolytic product was [^{32}P]lysolecithin. Subsequent to the preferential removal of [^{32}P]lecithin, we observed a relative enrichment of the VLDL with [^{32}P]sphingomyelin, and the ratio between these two phospholipids approached that found in LDL, the final product lipoprotein of VLDL degradation (2).

Several reports have shown that the plasma postheparin lipolytic activity is composed of at least two species of enzymes of hepatic and extrahepatic origin (24–29). The two species of enzymes can be assayed separately by preincubation of the postheparin plasma with protamine sulfate or 0.5 M NaCl, which predominantly inhibit the extrahepatic enzyme (24, 29), and the extrahepatic enzyme can be obtained free of the hepatic enzyme from the supradiaphragmatic portion of the rat (24). The tissue origin of plasma postheparin phospholipase was investigated by Zieve and Zieve (30) and Pykalisto, Vogel, and Bierman (31). Both studies indicate that the liver is a major source of the enzyme. Indeed, a hepatic lipase purified from postheparin plasma was shown recently to have considerable phospholipase activity (32). Heparin-releasable and nonreleasable phospholipase activity, however, can be found also in muscle and adipose tissue extracts (31). The present study demonstrates definitely that the extrahepatic enzyme obtained from the supradiaphragmatic portion of the rat can hydrolyze glycerophospholipids from a lipoprotein substrate, VLDL. Phospholipase activity is also associated with purified bovine milk lipoprotein lipase (an enzyme of extrahepatic characteristics (33, 34), and has been detected recently in a lipoprotein lipase isolated from the perfused rat heart.[3]

Whether the observations reported here occur during the physiological process of VLDL (and chylomicron) degradation is unknown. Phospholipids are

[2] Eisenberg, S., and T. Chajek. Unpublished observations.

[3] Chajek, T., and S. Eisenberg. Unpublished observations.

removed promptly from the density range of VLDL ($d < 1.006$ g/ml) after activation of the lipoprotein lipase by heparin injection to humans (7, 35) or rats (36). Lysolecithin accumulation was variable in these experiments, possibly reflecting the rapid extraction of lysolecithin from the plasma by tissues (37) and blood vessels (38). Thus, the possibility that during degradation of VLDL in situ, some, or most, of the VLDL lecithin is transferred directly to tissue cells without being hydrolyzed cannot be ruled out. An indication, however, that this may not be a predominant mechanism for removal of VLDL phospholipids is derived from our experiments carried out with whole blood, where we did not find a considerable transfer of ^{32}P-labeled phospholipids from VLDL to blood cells. It is therefore tempting to hypothesize that lecithin hydrolysis occurs also in situ and it may be a very early event of VLDL degradation. According to this hypothesis, phospholipid hydrolysis may begin even prior to triglyceride hydrolysis, thus exposing the neutral-lipid core to the enzyme. The continued phospholipase activity may thus facilitate the further hydrolysis of triglycerides by the endothelial-bound lipoprotein lipases and preserve a constant ratio of "surface" (polar) lipids to "core" (nonpolar) lipids (21, 23, 36, 39, 40). These hypotheses remain to be tested.

APPENDIX

When phospholipid specific activities and amounts of phospholipid radioactivity in VLDL are known, the ratio between these two values measures the reciprocal of the mass of phospholipids in VLDL. When plotted against the amount of radioactivity remaining in VLDL, the resulting values reflect the magnitude of exchange as against net transfer of phospholipids from VLDL. In an exchange reaction the ratio will be 1.0 regardless of the number of molecules exchanging. For example, if 80% of the labeled molecules have been replaced by 80% of unlabeled molecules, then the specific activity will be 0.2 of that found originally, as will be the amount of radioactivity in VLDL. However, when labeled molecules are being removed from the VLDL, then this ratio increases following the equation $1/(1 - x)$, where x is the fraction of phospholipids that have been removed. For example, if 80% of the labeled molecules have been removed (and not replaced by unlabeled molecules) then the specific activity will remain 1; however, the amount of radioactivity in VLDL will be 0.2 $(1 - 0.8)$ and the ratio of specific activity to amount of radioactivity will be 5.

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