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Accelerated clearance of low-density and high-density lipoproteins and retarded clearance of E apoprotein-containing lipoproteins from the plasma of rats after modification of lysine residues

(lipoprotein catabolism/protein modification/hepatic uptake/liver metabolism)

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ABSTRACT **Selective chemical modification of lysine residues of lipoproteins by acetoacetylation dramatically altered the metabolism of the lipoproteins without significantly altering other physical or chemical properties. Modification of 30–60% of the total lysine residues of iodinated rat or human low-density lipoproteins (^{125}I-LDL) resulted in a rapid removal of these acetoacetylated lipoproteins from the plasma of rats. Within minutes after intravenous injection into intact rats, greater than 80% of the total injected dose disappeared from the plasma. The rapidly cleared acetoacetylated LDL appeared in the liver, and within 6–30 min as much as 50–80% of the total injected dose of modified LDL could be accounted for in the liver. Furthermore, it was possible to demonstrate in the isolated perfused rat liver that the Kupffer cells were responsible for the lipoprotein uptake. Human high-density lipoproteins (HDL_3) were also rapidly removed from the plasma after acetoacetylation. In striking contrast, acetoacetylation (30–60%) of two E apoprotein-containing lipoproteins (rat HDL_1 and dog HDL_c) retarded their removal from the plasma. The accelerated removal of modified LDL and HDL_3, in contrast to the retarded removal of modified HDL_1 and HDL_c, suggests that the recognition and removal process is specific for a property acquired by only certain lipoproteins after acetoacetylation. Moreover, these results suggest that lysine residues of the E apoprotein may play a functional role in the recognition process for the normal clearance of HDL_1 and HDL_c, a process that is interfered with after acetoacetylation.**

The apoprotein constituents of plasma lipoproteins have been shown to be involved in the control of various aspects of lipoprotein metabolism. For example, it has been demonstrated that the recognition site on lipoproteins responsible for their binding to the cell surface receptors of fibroblasts in culture resides with specific apoproteins (1–4). Both the B apoprotein of low-density lipoproteins (LDL) and the E apoprotein of certain high-density lipoproteins (HDL_1, HDL_c) react with the high-affinity receptors. Procedures for chemical modification of specific amino acid residues without irreversible alteration of the chemical or physical properties of the lipoproteins have been developed and constitute an approach to probing the role of these residues in apoprotein metabolism (2, 4). The modification of a limited number of arginyl and lysyl residues of the lipoproteins that bind to the cell surface receptor has been shown to abolish the ability of the lipoproteins to bind to these receptor sites (2, 4).

In this study we examined the effects of lysine modification, by acetoacetylation, on the metabolism *in vivo* of various lipoproteins in the rat, an animal suitable for metabolic studies with heterologous lipoproteins (5). Acetoacetylation is highly specific for modification of lysine residues under the conditions previously described (4). Furthermore, acetoacetylation can be reversed to regenerate a native lipoprotein with properties virtually identical to those of the unmodified lipoprotein (4). The lipoproteins investigated in the present study included human and rat LDL containing the B apoprotein, human HDL containing primarily the A-I and A-II apoproteins, and the rat and canine lipoproteins (HDL_1 and HDL_c) containing the E apoprotein. The different effects of lysine modification on the removal of these plasma lipoproteins from the plasma will be described.

MATERIALS AND METHODS

Isolation and Iodination of Lipoproteins. Human LDL (density 1.02–1.05 g/cm^3) and HDL_3 (density 1.125–1.21 g/cm^3) were obtained from the plasma of a fasted male subject by sequential ultracentrifugation at 59,000 rpm in a 60 Ti rotor (Beckman). The LDL were obtained after 18 hr of centrifugation and washed at density 1.050 g/cm^3 with an additional centrifugation. The HDL_3 were isolated and washed by centrifugation for 36 and 24 hr, respectively. Rat LDL and HDL_1 (density 1.02–1.063 g/cm^3) were isolated by ultracentrifugation of plasma from fasted rats and purified by Geon-Pevikon block electrophoresis as described (6). Canine apo-E HDL_c was obtained by Geon-Pevikon block electrophoresis of the 1.006- to 1.02-g/cm^3 ultracentrifugal fraction from plasma of foxhounds fed a semisynthetic diet containing coconut oil and cholesterol (7). Human LDL and HDL_3 were iodinated by the iodine monochloride method (8). The rat LDL and HDL_1 and the canine HDL_c were iodinated by the Bolton-Hunter method (9). Sodium [^{125}I]iodide (carrier free) and ^{125}I-labeled Bolton-Hunter reagent were purchased from Amersham/Searle.

Acetoacetylation of Lipoproteins. The lysine residues of the ^{125}I-labeled lipoproteins were modified by acetoacetylation with freshly distilled diketene (Sigma) as described in detail previously (4). To each milligram of lipoprotein protein in 0.1 M sodium borate buffer, pH 8.5, 0.2–4.0 μmol of diketene was added and the reaction was allowed to proceed for 5 min at 26°C. The reaction was stopped by dialysis against 0.2 M carbonate/bicarbonate buffer, pH 9.5. The amount of diketene reagent required was determined empirically by measuring the extent of acetoacetylation for each lipoprotein (4). Reversal of the acetoacetylation was accomplished by incubating the modified lipoproteins with 0.5 M hydroxylamine for 16 hr, as

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Abbreviations: LDL, low-density lipoproteins; HDL, high-density lipoproteins; apo-E, arginine-rich apoprotein.

described (4). The extent of lysine modification was determined by the trinitrobenzenesulfonic acid colorimetric assay (10) and by amino acid analysis (4). The colorimetric assay consistently gave values that were 7% higher than those obtained by amino acid analysis. All results are reported on the basis of amino acid analysis. All control and modified lipoproteins were characterized by paper electrophoresis, apoprotein content by gel electrophoresis, and particle morphology by negative staining electron microscopy (4, 7).

***In Vivo* Studies.** Male Sprague–Dawley rats (200–250 g) were lightly anesthetized with ether and injected via an exposed saphenous vein with the ^{125}I-labeled lipoproteins at a dose of 100–300 μg of protein. Each rat used in the study was weighed just prior to injection, and the plasma volume of each rat was estimated as 4% of the body weight. At specified time intervals after lipoprotein injection, the rats were exsanguinated through the abdominal aorta. The liver and spleen were removed, washed with saline, and weighed. Slices (50–200 mg) of the washed organs were weighed and digested in Protosol (New England Nuclear), and the radioactivities were measured. All values represent duplicate determinations.

Isolated Liver Perfusion. Isolated livers of 275-g male Osborne–Mendel rats (fasted overnight) were perfused with 70 ml of recycled perfusate (20.4 ml/min flow; 37°C) by the method of Mortimore (11). The perfusate consisted of 20% (vol/vol) washed rat erythrocytes in Krebs–Ringer bicarbonate buffer, pH 7.4, which contained 0.2% glucose, 5% bovine serum albumin (Fraction V, Armour), streptomycin sulfate at 10 mg/100 ml, and penicillin G at 200,000 units/100 ml. The perfusate was continuously equilibrated with 95% O_2/5% CO_2. The labeled lipoproteins were added 10 min after the recycling was started and the perfusion was terminated 17.5 min later. The livers were flushed without interruption of flow with unlabeled perfusate at 37°C (51 ml), then with 0.15 M NaCl/0.1% glucose/0.2% bovine serum albumin at 4°C (50 ml), and finally with 3% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, at 4°C (50 ml). Sections of the fixed livers were obtained for measurement of radioactivity and for microscopy. Autoradiography was performed on 1-μm Epon-embedded sections with NTB-2 Kodak emulsion. Sections were stained with methylene blue/azure II.

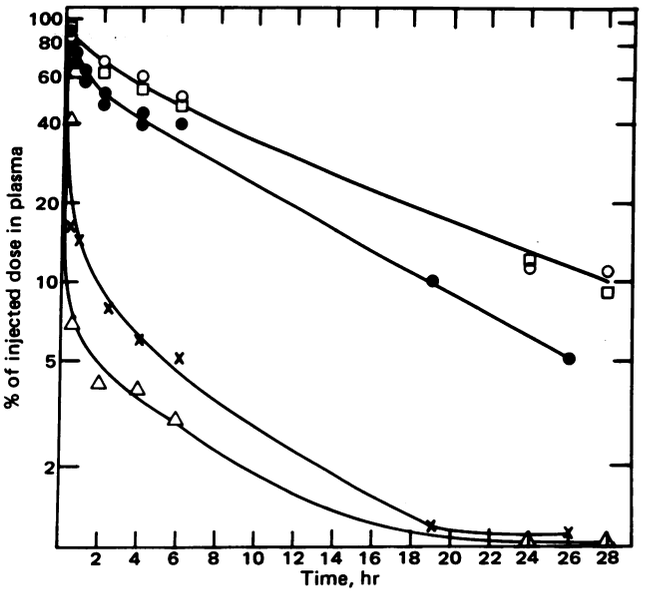

FIG. 1. *In vivo* removal from rat plasma of ^{125}I-labeled control rat LDL (●), control human LDL (○), acetoacetylated rat LDL (x) that had 35% of the lysine residues modified, acetoacetylated human LDL (△) that were 60% modified, and acetoacetylated–reversed human LDL (□).

RESULTS

Rat and human LDL have similar rates of disappearance from the plasma when injected intraveneously into rats. Approximately 50% of the total injected dose of the rat and human ^{125}I-LDL remained in the plasma after 4–6 hr (Fig. 1, Table 1). However, modification of the lysine residues of the LDL by acetoacetylation resulted in a rapid clearance of these lipoproteins from the plasma; e.g., more than 80% of the total in-

Table 1. Percent distribution of the total injected dose of control and modified ^{125}I-LDL in the rat

Time, min	Human LDL control				Human 60% modified*				Human modified–reversed†			
	Plasma	Liver	Spleen	Total	Plasma	Liver	Spleen	Total	Plasma	Liver	Spleen	Total
6	87.4	9.6	0.3	97.3	41.0	54.0	0.5	95.4	97.7	10.0	0.3	108.0
30	80.9	6.2	0.2	87.3	7.3	22.1	1.2	30.6	82.8	11.3	0.4	94.4
120	68.1	11.0	1.3	80.3	4.2	10.9	1.0	16.1	62.9	16.6	1.3	80.8
240	60.1	8.7	0.7	69.5	4.3	7.8	0.7	12.8	51.9	8.9	0.7	61.5
360	49.6	5.9	0.5	55.9	2.8	4.8	0.3	7.9	44.8	6.1	0.6	51.4
1440	11.1	2.2	0.3	13.5	0.6	2.3	0.2	3.1	12.0	2.9	0.3	15.2
1800	11.3	2.6	0.3	14.2	0.6	1.9	0.1	2.6	7.0	1.9	0.3	9.1
	Rat LDL control				Rat 35% modified‡				Rat LDL control§			
5	90.0	13.0	0.2	103.2	70.8	28.5	0.7	100.0	—	—	—	—
30	74.1	14.7	0.4	89.2	18.1	49.0	0.8	68.0	75.1	15.4	0.5	91.0
60	58.3	21.2	0.2	79.7	15.1	23.9	0.7	39.6	64.7	14.2	0.5	79.4
120	48.4	14.1	0.3	62.8	8.4	7.0	0.2	15.6	49.6	12.7	0.4	62.7
240	45.3	8.4	0.2	53.9	6.1	2.8	0.2	9.1	42.2	9.4	0.3	51.9
360	40.0	6.6	0.2	46.8	5.3	2.5	0.1	7.9				
1140	10.3	2.1	0.1	12.4	1.5	1.1	0.1	2.7				
1560	5.0	1.4	0.1	6.5	1.2	1.0	0.1	2.3				

* Acetoacetylation of the human LDL modified 60% of the total lysine residues.

† Acetoacetylation of 60% of the total lysine residues was reversed by incubation with hydroxylamine. Less than 1% of the lysine residues remained modified after the reversal.

‡ Acetoacetylation of the rat LDL modified 35% of the total lysine residues.

§ An additional short-term study.

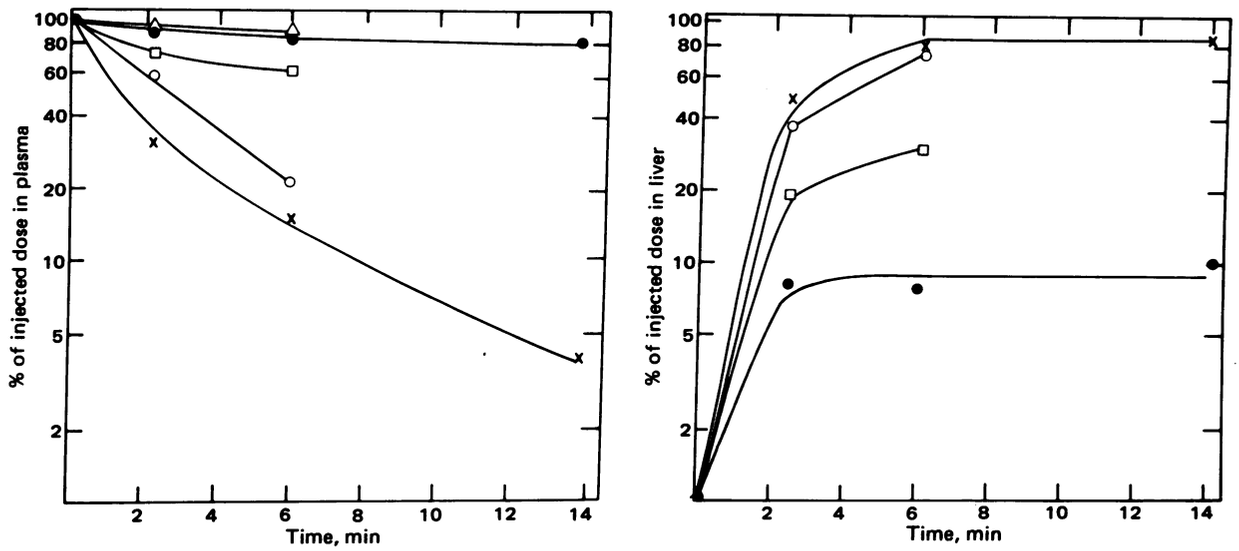

FIG. 2. Percent of the total injected dose of ^{125}I-labeled human LDL that remained in the plasma (*Left*) and that appeared in the liver (*Right*) immediately after the injection. Control LDL (●) were compared to acetoacetylated LDL at various levels of lysine modification: 27% (△), 37% (□), 48% (○), and 68% (×). Each rat was injected with 160 μg of lipoprotein protein.

jected dose was removed within 30 min (Fig. 1). In this particular study 35% and 60% of the total lysine residues of the rat LDL and human LDL, respectively, had been acetoacetylated. The acetoacetylated ^{125}I-LDL rapidly appeared in the liver and as much as 50% of the total activity injected could be accounted for in the liver within 5–30 min (Table 1). By comparison, the activity in the liver of rats that received control LDL was seldom in excess of 10% of the total injected dose at any time interval studied. The activity within the spleen usually represented less than 1% of the injected radioactivity of either the control or modified LDL. The results obtained with control human LDL in rats are in agreement with those previously reported (5).

It was possible to reverse almost completely the acetoacetylation of the lysine residues of the LDL, as previously described (4), and to restore to the LDL a metabolic activity essentially identical to that of control LDL. Human LDL that had been acetoacetylated (60% of the lysines modified) and then regenerated by reversal of the modification (1% of lysines remained modified) were not rapidly cleared and, in fact, had a disappearance from the plasma (Fig. 1) and a hepatic uptake (Table 1) very similar to the pattern observed with the control LDL. These results demonstrate that the acetoacetylation does not irreversibly alter the lipoproteins. Furthermore, except for an increased electrophoretic mobility that reflected the neutralization of the positive charge on the lysine residues, the modified LDL did not exhibit any alteration in lipid composition, apoprotein content as determined by gel electrophoresis, or particle size or morphology by negative staining electron microscopy. The mild conditions of the reaction and its almost complete reversibility have been discussed (4).

The rapid uptake of the acetoacetylated human LDL after the injection into rats was verified in short-term studies (Fig. 2). During the first 6 or 14 min, it was possible to account for 82–100% of the total injected dose in the plasma plus the liver for both control and modified LDL. For example, as shown in Fig. 2, 95% of the acetoacetylated ^{125}I-LDL (68% of the lysines modified) was recovered after 6 min, with 16% of the activity in the plasma and 79% in the liver. At the same time point, 84% of the activity of the control LDL remained in the plasma and only 7% was in the liver (representing 91% of the total injected ^{125}I-LDL).

Furthermore, in these acute studies there was a relationship between the percentage of the total lysine residues modified and the rate of clearance from the plasma and appearance in the liver. As shown in Fig. 2, the extent of the rapid disappearance of LDL from the plasma increased progressively as the level of the chemical modification of the lysine residues was increased from 37% to 68%. Likewise, the appearance of activity in the liver was increased. However, modification of only 17% (data not shown) or 27% (Fig. 2) of the total lysine residues of human ^{125}I-LDL did not alter their removal rate. In several studies, we found that modification of less than 30% of the lysine residues did not accelerate the removal of LDL immediately or for up to 24 hr. It is noteworthy that all the modified LDL used in this study, including those that were 17% and 27% modified, were incapable of binding to the cell surface receptors of human fibroblasts as determined by direct binding and degradation assays. Previously, we reported (4) that acetoacetylation of 20% or more of the lysine residues of LDL abolished receptor binding activity.

The rapid uptake of the modified LDL was found to be a

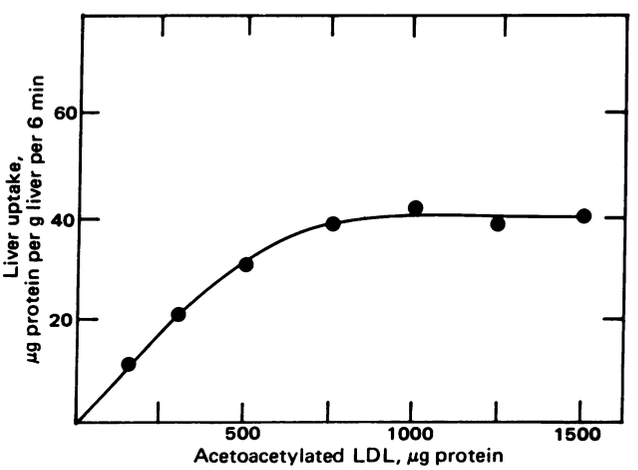

FIG. 3. Hepatic uptake (μg of protein per g of liver per 6 min) of acetoacetylated human ^{125}I-LDL (60% of the lysine residues modified) vs. the concentration of acetoacetylated LDL injected (μg of protein per 250-g rat).

saturable process. At dose levels in excess of 750 μg of modified ^{125}I-LDL protein, there was no additional accumulation of activity in the liver in the 6-min time interval (Fig. 3). How quickly the saturated system recovers remains to be determined. At the nonsaturating levels of LDL used in the various studies, it was possible to compare the amounts of control and modified LDL removed by the liver from the plasma at 6 min. Results, compiled in Table 2 for two experiments, reveal that 4–8 times more acetoacetylated LDL, as compared to control LDL, were taken up by the liver.

To identify the cell type in the liver responsible for the rapid uptake of the acetoacetylated LDL, modified human LDL were perfused through the isolated rat liver for 17.5 min. At the end of the study, the liver was perfused extensively with unlabeled perfusate and buffer until essentially no activity was detected in the effluent fluids. Then the liver was fixed by perfusion with glutaraldehyde. The liver accumulated 56 μg of ^{125}I-LDL protein per gram of liver during the 17.5-min perfusion. As a control, a liver was perfused with human LDL that had been modified and then regenerated by reversal of the modification. The control liver accumulated 7 μg of ^{125}I-LDL protein per gram of liver in 17.5 min. The reversed-modified LDL had a disappearance *in vivo* identical to that of control LDL. Autoradiography on the liver perfused with the acetoacetylated LDL demonstrated extensive labeling in the sinusoidal cells (Kupffer cells) and only an occasional silver grain over parenchymal cells (Fig. 4). The liver perfused with the reversed-modified LDL had much less activity, but visible silver grains were associated primarily with parenchymal cells and were only rarely associated with the sinusoidal space or lining cells.

Acetoacetylation of human HDL_3 (density 1.125–1.21 g/cm^3) accelerated the clearance of this lipoprotein, with appearance of the label in the rat liver. However, the disappearance from the plasma was never as rapid as observed with LDL. In one study in which 90% of the lysine residues of the HDL_3 were modified, 55% of the injected dose of acetoacetylated HDL_3 was in the plasma and 43% was in the liver after 6 min. At 30 min, 45% of the modified HDL_3 remained in the plasma. Although removal of HDL_3 from the plasma was not as dramatic as with LDL, it was greatly accelerated by comparison with the disappearance of control HDL_3. Greater than 90% of the control HDL_3 remained in the plasma 30 min after injection. Reversal of the acetoacetylation of the HDL_3 by hydroxylamine treatment restored to these lipoproteins a disappearance *in vivo* identical to that of the control HDL_3. The only property of the HDL_3 altered by acetoacetylation was an increased electrophoretic mobility, which was also reversed by hydroxylamine treatment.

Table 2. Hepatic uptake of control and modified ^{125}I-LDL after 6 min in the rat*

	μg protein per g liver	
	Exp. I†	Exp. II‡
Control LDL	1.1	1.4
Modified (60%)–reversed	1.0	1.2
Modified LDL§		
27%	—	0.9¶
37%	—	4.4
48%	—	9.4
50%	4.4	—
60%	4.7	—

* Of the total activity injected, 95–100% was recovered in the plasma plus the liver at 6 min.
† LDL protein injected per rat, 100 μg.
‡ LDL protein injected per rat, 160 μg.
§ Following entries are percent of total lysine residues modified.
¶ The removal of this lipoprotein from the plasma was identical to that of control LDL (Fig. 2).

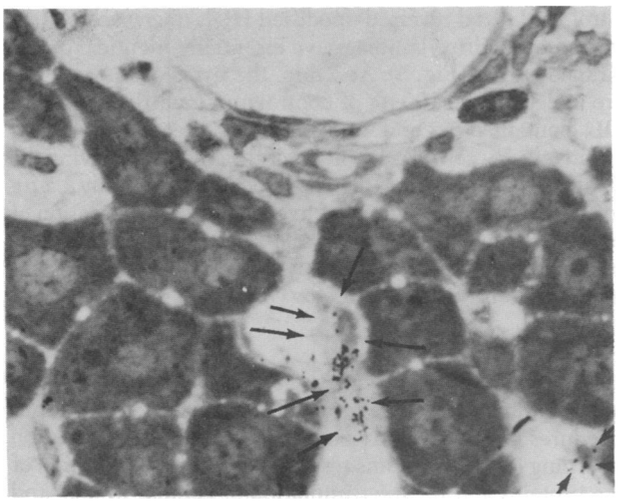

FIG. 4. Autoradiograph of a section of rat liver perfused with acetoacetylated human LDL. The silver grains are primarily associated with the sinusoidal lining cells (arrows). (×860.)

In striking contrast to the enhanced clearance of modified LDL and HDL_3, acetoacetylation retarded the removal of E apoprotein-containing lipoproteins from the plasma. Two lipoproteins that contain primarily or exclusively the E apoprotein are rat HDL_1 and cholesterol-induced canine HDL_c (6, 7). Rat HDL_1 have been defined as HDL-like lipoproteins isolated in an ultracentrifugal density range that overlaps those of LDL and HDL (density 1.03–1.08 g/cm^3). The HDL_1 contain the E apoprotein as a major constituent (estimated >80% of total protein), variable amounts of the A-I and C apoproteins, and no B apoprotein (6). The cholesterol-induced canine HDL_c contain the E apoprotein as the only detectable protein constituent (7). As shown in Fig. 5, acetoacetylation of rat HDL_1 and canine HDL_c did not accelerate their removal from the plasma but instead retarded it. Lipoproteins used in this study were modified to differing extents, which ranged from 32% (rat HDL_1) to 57% (canine HDL_c) of the total lysine residues. The

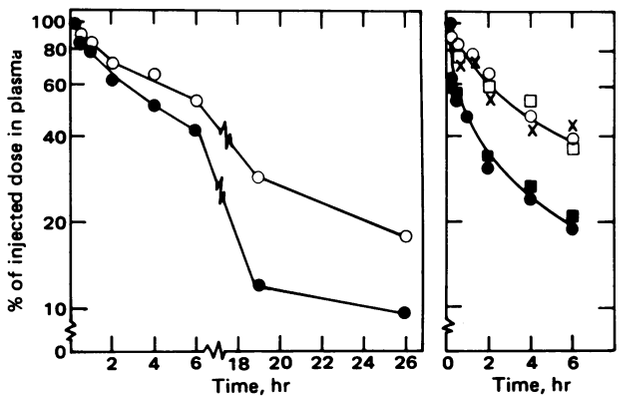

FIG. 5. Percent of the total injected dose of ^{125}I-labeled lipoprotein that remained in the plasma. (*Left*) Control rat HDL_1 (●) were compared to acetoacetylated rat HDL_1 (32% of the lysines modified). (*Right*) Cholesterol-induced control dog HDL_c (●) were compared to HDL_c modified to different extents: 38% (○), 54% (□), and 57% (×). The acetoacetylated–reversed canine HDL_c (■) gave results similar to those of control HDL_c (●). Each rat was injected with 100 μg of lipoprotein protein.

control HDL_c and reversed-modified HDL_c (acetoacetylation reversed by hydroxylamine) gave essentially identical disappearance curves (Fig. 5). At 5 min, the livers of the rats that were injected with 100 μg of control and modified-reversed HDL_c contained 5.2 and 4.6 μg of HDL_c per gram of liver, respectively. By contrast, the livers of the animals receiving the 38% and 57% acetoacetylated HDL_c accumulated only 1.2 μg of modified HDL_c per gram of liver in 5 min. Retardation of the hepatic removal of canine apo-E HDL_c after acetoacetylation of more than 30% of the lysine residues has been confirmed in extensive studies *in vivo* conducted in dogs (unpublished data).

DISCUSSION

Studies with plasma proteins chemically modified by various procedures have provided potentially important information concerning the mechanisms responsible for the recognition of these proteins and for the stimulation of cellular uptake and catabolism. For example, receptor-mediated hepatic uptake of certain plasma proteins can be stimulated by removal of *N*-acetylneuraminic acid (12). With other proteins, covalent attachment of sugars and polysaccharides alters the rates of removal (13). Little information is available concerning the control of catabolism of plasma lipoproteins. However, the present study demonstrates that acetoacetylation of 30–60% of the lysine residues of LDL results in a markedly accelerated removal of these lipoproteins from the plasma and their uptake by the liver, primarily by Kupffer cells. The uptake process is saturable and the perfused liver studies show that the accelerated hepatic uptake does not require the intervention of any other organ.

Acetoacetylation does not grossly alter known physical or chemical properties of the LDL except for the expected change in their electrophoretic mobility indicative of a neutralization of the positive charge on the modified lysine residues (4). Furthermore, the reversibility of the acetoacetylation and the regeneration of a lipoprotein with properties indistinguishable from those of the control LDL support the conclusion that extensive alteration has not occurred. Whether or not the uptake is stimulated as a direct response to the altered charge is open to speculation.

However, the striking difference between the enhanced removal of the acetoacetylated LDL and HDL_3 and the retarded removal of the acetoacetylated apo-E-containing HDL_1 and HDL_c suggests an apoprotein-mediated specificity of the removal process. As with LDL, the only change observed in the HDL_1 and HDL_c after acetoacetylation was an increased electrophoretic mobility secondary to neutralization of the positive charge on the lysine residues (4). It is reasonable to speculate that the decrease in the rate of plasma clearance of modified HDL_1 and HDL_c reflects an interference with a receptor-mediated uptake process in which lysine residues of the E apoprotein are involved. Furthermore, the reduction in the amount of HDL_c that appears in the liver after modification of the lysine residues of the E apoprotein suggests that the hepatic recognition process may be mediated by the E apoprotein, a process that is interfered with after acetoacetylation.

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