Side-chain oxidation of lipoprotein-bound [24,25-^{3}H]cholesterol in the rat: comparison of HDL and LDL and implications for bile acid synthesis

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Abstract The purpose of the study was to test the hypothesis that high density lipoprotein (HDL) cholesterol would be more easily oxidized in vivo than low density lipoprotein (LDL) cholesterol. Homologous plasma was incubated with [24,25-^{3}H]cholesterol and fractionated by ultracentrifugation to obtain HDL and LDL each labeled with [^{3}H] free sterol. HDL and LDL labeled with [24,25-^{3}H]cholesteryl esters were prepared by ultracentrifugation of plasma from donor rats injected 24 hr previously with [24,25-^{3}H]cholesterol in propylene glycol. These four labeled lipoproteins were administered to recipient rats. It was found that more tritium oxide (^3H_2O) was produced after the HDL doses than after the corresponding LDL doses, from 2–3-fold more when lipoprotein free cholesterol was labeled and from 2–6-fold more when lipoprotein cholesteryl esters were labeled. More ^3H_2O was produced from free cholesterol-labeled lipoproteins than from cholesteryl ester-labeled lipoproteins. Since oxidation of cholesterol is a measure of bile acid formation, it is concluded that under the conditions of the study HDL-cholesterol is a better precursor of bile acids than LDL-cholesterol.—**Miller, L. K., M. L. Tiell, I. Paul, T. H. Spaet, and R. S. Rosenfeld.** Side-chain oxidation of lipoprotein-bound [24,25-^{3}H]cholesterol in the rat: comparison of HDL and LDL and implications for bile acid synthesis. *J. Lipid Res.* 1982. **23:** 335–344.

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The inverse relationship between high density lipoprotein (HDL) levels and the incidence of coronary artery disease has fostered increased study into possible underlying mechanisms. Several lines of experimental evidence suggest that the beneficial effect of HDL may be associated with the transport of cholesterol from tissue sites. In culture systems using smooth muscle cells (1–3), endothelial cells (4, 5), fibroblasts (6–8), or macrophages (9, 10), HDL facilitates the removal of cholesterol from the cells. Further, it has been hypothesized from circumstantial evidence that HDL is instrumental in transporting cholesterol to the liver (11, 12) where metabolic processes involving HDL occur five to six times faster than with LDL (13, 14). Schwartz and co-workers (15) showed in a patient with a bile fistula that biliary excretion of radioactive cholesterol was greater when HDL carried the labeled sterol. Using a similar approach, Portman, Alexander, and O'Malley studied the metabolism of HDL- and LDL-bound free and esterified cholesterol in squirrel monkeys and obtained similar results (16). Related investigations involving hepatic clearance of lipoproteins in the cebus monkey (17) and uptake of HDL-cholesterol across the splanchnic bed of the baboon (18) afforded results which are in accord with the preferential utilization of HDL-cholesterol over LDL-cholesterol by the hepato-biliary system.

Since a significant portion of cholesterol is converted to bile acids in vivo, it has been reasoned that the HDL-cholesterol might be transformed to bile acids in preference to LDL-cholesterol. Halloran et al. (19) administered HDL and LDL, each containing cholesterol with a different label, to a patient with a T-tube drainage of the bile duct and found that the bile acids derived from HDL-cholesterol contained proportionally more of that isotope. With a similar protocol, Portman and his colleagues (16) confirmed the work of Schwartz, Halloran, and their associates (15, 19). Thus, increased concentrations of HDL could well be associated with increased catabolism of cholesterol, explaining in part the putative anti-atherogenic properties of this class of lipoproteins.

However, not all experimental evidence is consonant with the preferential utilization of HDL-cholesterol for bile acid formation. Sniderman et al. (20) reported that LDL-cholesterol showed a decrease across the splanchnic bed in man while HDL-cholesterol concentration did not

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; FC, free cholesterol; EC, cholesteryl esters; HDL tracer (FC), tracer dose, [24,25-^{3}H]cholesterol-HDL; HDL tracer (EC), tracer dose [24,25-^{3}H]cholesteryl ester-HDL; LDL tracer (FC), tracer dose [24,25-^{3}H]cholesterol-LDL; LDL tracer (EC), tracer dose, [24,25-^{3}H]cholesteryl ester-LDL.

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change, and in hypercholesterolemic subjects, Nestel and Billington (21) observed that treatment with Probucol® was associated with increased fractional removal rate of [^{131}I]-labeled LDL accompanied by an increase in bile acid excretion. In rat liver perfusion studies, the catabolic rate of HDL was much lower than that measured in vivo, suggesting that a major portion of HDL could be metabolized peripherally (22).

Previous studies in our laboratory demonstrated that the formation of tritium oxide (^3H_2O) from [24,25-^{3}H]cholesterol is a measure of bile acid biosynthesis (23). In view of the preferential utilization of HDL-cholesterol for bile acid production (17, 19), we reasoned that [24,25-^{3}H]cholesterol bound to HDL would be more rapidly oxidized to bile acids than [24,25-^{3}H]cholesterol bound to LDL, and that this would be observed in the intact animal by a greater rate of appearance of ^3H_2O in the circulation. Further, depending on whether [24,25-^{3}H]cholesterol was free or esterified, tracer lipoproteins could be prepared in which the label was present at the surface or within the core of the particle so that both types could be studied as substrates for in vivo oxidations. Using these tracers as such, or in conjunction with other labeled lipoproteins, to measure bile acid formation without preparation of bile fistulas or tedious isolation from feces, could permit the use of simpler experimental models in investigating the metabolism of cholesterol under a variety of experimental conditions.

EXPERIMENTAL

Preparation and purification of [24,25-^{3}H]cholesterol

The side-chain labeled sterol was prepared from desmosterol (5,24-cholestadien-3β-ol) as previously described (23). Briefly, the Δ^5 double bond was protected by converting the starting substance to 3,5-cyclocholest-24-en-6β-methyl ether, which was then catalytically reduced at C-24,25 with tritium by the Tritium Labeling Service of New England Nuclear Corp., Boston, MA. The tritiated product was returned to this laboratory and reconverted to the Δ^5-3β-ol. [24,25-^{3}H]Cholesterol was purified to radiochemical homogeneity by bromination-debromination procedures, alumina chromatography, and recrystallization (23). Oxidation of a sample to 3β-hydroxy-5-androsten-17-one demonstrated that over 98% of the radioactivity is located in the side-chain. Furthermore, the isolation of bile acids devoid of radioactivity after administering the tracer to subjects demonstrated that the labeling in the side-chain is restricted to the terminal four carbons. [24,25-^{3}H]Cholesterol was stored in benzene–methanol solution at 4°C until used. After 6–10 months storage, the material was 60–80% pure and was brought to >95% radiochemical purity by several recrystallizations from acetone before administration.

Preparation of lipoproteins labeled with [24,25-^{3}H]cholesterol or [24,25-^{3}H]cholesteryl esters

In vitro labeling with tritiated free cholesterol. The labeling procedures were essentially those of Schwartz et al. (24). About 0.75 g of 5 mm diameter Whatman #1 filter paper discs were placed in a siliconized 125-ml Erlenmeyer flask to which was added about 3 mCi of [24,25-^{3}H]cholesterol (approximately 3 mg) in benzene. The solution was added in quantities sufficient to wet the filter paper, and the solvent was evaporated under nitrogen. About 15 ml of plasma from donor rat blood, anticoagulated with EDTA and centrifuged at 4°C, was poured onto the impregnated filter paper discs and the mixture was shaken for 4 hr under nitrogen in an ice-bath. The labeled plasma was decanted and centrifuged in the cold to remove filter paper fibers.

In vivo labeling with tritiated cholesteryl esters. Male Sprague-Dawley rats weighing 350–400 g were anesthetized with ether and injected via the femoral vein with about 1 mCi of [24,25-^{3}H]cholesterol (~1 mg) in 0.5 ml of propylene glycol–ethanol (9:1) per rat; the rats were kept for 24 hr in individual cages. The animals were then re-anesthetized with ether and killed by exsanguination via a 19-gauge butterfly needle placed into the abdominal aorta. About 15 ml of free-flowing blood was collected from each donor into tubes containing EDTA anticoagulant. The samples were cooled and centrifuged at 4°C to yield plasma in which the major portion of the radioactivity was present in cholesteryl esters. To remove any labeled free cholesterol, the plasma was incubated with non-radioactive rat erythrocytes (6:4, v/v) for 3 hr at 37°C (25); following centrifugation, the incubation was repeated for an additional 3 hr with fresh rat red cells. After a second centrifugation, the plasma was used immediately or stored at −70°C for no more than 72 hr before fractionation.

Lipoprotein fractionation. All the procedures were carried out between 0–4°C. Plasma was processed essentially as described by Hojnacki et al. (26). Briefly, 4 ml of the labeled plasma was overlayered with 2 ml of a solution consisting of 3 mM sodium azide (NaN_3), 0.3 mM disodium EDTA (Na_2EDTA), and NaCl ($\rho_{25°C}$, 1.007 g/ml), and centrifuged at 1.15×10^5 *g* for 16 hr in a Beckman L2-65B preparative ultracentrifuge using a fixed angle rotor (Beckman Ti-40.3). The upper 2 ml of solution containing chylomicrons and VLDL was discarded and the infranate was mixed with 2 ml of a solution of NaBr in NaN_3–Na_2 EDTA ($\rho_{25°C}$, 1.190 g/ml), and centrifuged under the same conditions for 24 hr. The resulting upper 1 ml of supernatant containing the LDL was saved and a second 1-ml portion imme-

diately below was removed and discarded. The LDL fraction was dialyzed overnight against a large volume of 150 mM NaCl–0.3 mM Na_2EDTA. The density of the remaining 4 ml of infranate was adjusted to 1.215 g/ml by adding 2 ml of NaBr–NaN_3–Na_2EDTA ($\rho_{25°C}$, 1.509 g/ml) and the mixture was centrifuged as above for at least 40 hr. The upper 1 ml, the HDL, was removed and dialyzed as described for the LDL. The dialyzed LDL and HDL fractions, containing either [24,25-^{3}H]cholesterol or [24,25-^{3}H-]cholesteryl esters, depending on the method of labeling, were then immediately administered to the experimental animals (recipients).

Analytical gel filtration of the labeled LDL and HDL

An aliquot (250 μl) of LDL or HDL labeled with [24,25-^{3}H]cholesterol or [24,25-^{3}H]cholesteryl ester was mixed with ^{14}C-labeled standard proteins (27, 28), [^{14}C]urea, and 250 mM sucrose in column buffer and layered onto matched columns (1.3 × 95 cm) of Agarose A-5m, 200–400 mesh (Bio-Rad Laboratories, Richmond, CA). The columns were eluted at a flow rate of ~4 ml/hr with 10 mM Tris, pH 8.2 (25°C) containing 150 mM NaCl, 3 mM NaN_3, and 0.3 mM Na_2EDTA (29). Aliquots (0.8 ml) of the fractions (1.2 ml) were counted in 10 ml of ScintiVerse (Fisher Scientific Co., Fairlawn, NJ) in a liquid scintillation spectrometer (Tracor Analytic, Model 6892, Stanford, CT) and corrected for quench and isotope spillover by the external standard ratio method. The efficiencies in the two channels were approximately 35% and 0.001% for tritium, and 10% and 65% for ^{14}C. The void volume (V_0) of the column was estimated from the elution position of labeled lipoprotein aggregates or of blue dextran in studies where only standard proteins are used. The total column volume (V_t) was estimated from the elution position of [^{14}C]urea and the distribution coefficients (K_D) of lipoproteins and standard proteins were calculated as described by Gelotte (30). The molecular weights (M) and Stokes radii (R_S) of the lipoproteins were estimated from the regression of $M^{1/2}$ on $K_D^{1/3}$ (31) or log R_S on K_D (32) for the following standards: MS-2 virus (33), bovine thyroglobulin, rabbit muscle aldolase, human serum transferrin, and ovalbumin (M and R_S for standards other than MS-2 virus are from Table 1 of references 34 and 35).

Experimental protocol

Anesthesia, administration of the doses, and blood collection. Tritiated cholesterol– or cholesteryl ester–LDL or HDL were administered to anesthetized rats (recipients) via the femoral vein according to the procedure described above for preparation of the in vivo labeled lipoproteins. Blood (1.5–2.0 ml) was obtained from the tail vein at specified times up to 1 hr in the first study and 2 hr in the second. At the last collection the rat was anesthesized with ether and exsanguinated via the abdominal aorta; all blood was collected into EDTA, cooled in an ice-bath, and centrifuged at low speed to obtain the plasma. The plasma was stored at −70°C until processed, never longer than one day.

Procedural details–recipient plasma: distribution of radioactivity in free cholesterol and cholesteryl ester. Fifty microliters of plasma from each collection were mixed with 2 ml of acetone–ethanol 1:1 containing about 250 μg of carrier cholesterol and 750 μg of nonradioactive cholesteryl palmitate. The mixture was centrifuged and the supernatant was saved. The residue was washed once with acetone, then once with ether; the washings were combined with the supernatant and concentrated under nitrogen. The lipid residue was leached three times with hexane, and the hexane washings were combined and concentrated. Thin-layer chromatography of the nonpolar lipids on 250 μm layers of silica gel G (Brinkmann, Great Neck, NY) in the system ethyl acetate–isooctane 3:7 separates the free cholesterol and cholesteryl esters. The appropriate areas, located by spraying pilot spots with phosphomolybdic acid in ethanol, were scraped directly into scintillation vials for radioactive assay. This and subsequent counting was carried out in the single labeled mode with an efficiency of 45–50%. Aliquots of the labeled lipoprotein doses were treated identically.

Lyophilization. The remainder of the early small plasma samples and aliquots of the larger samples obtained at killing of the recipient rats were lyophilized. The lyophilizate, ^3H_2O, was diluted quantitatively to 1.0 ml for counting.

Specific activity of cholesterol in plasma lipoproteins obtained from recipient animals. Two milliliters of plasma were fractionated by the techniques described above to afford the HDL and LDL components. Each lipoprotein solution was made up to 10% NaOH in 70% ethanol and heated for 30 min at 75°C. After cooling, hexane was added to the tubes and the contents were well agitated. After separation of the layers, the alkaline solution was removed and the hexane layer was made up to exactly 5.00 ml in benzene, and 20% was removed for counting. The remaining 80% was assayed for cholesterol by GLC after the addition of cholestane as the internal standard; GLC on 3% QF-1-Gas Chrom Z (Supelco, Bellefonte, PA) at 256°C. Samples were counted in 10 ml or 18 ml of ScintiVerse as described above.

Radiochemical purity of [24,25-^{3}H]cholesterol in the administered lipoprotein doses. The radiochemical purity of the cholesterol in each labeled lipoprotein was checked by reverse isotope dilution procedures. A 50-μl aliquot of each fraction was added to 50 ml of acetone–ethanol

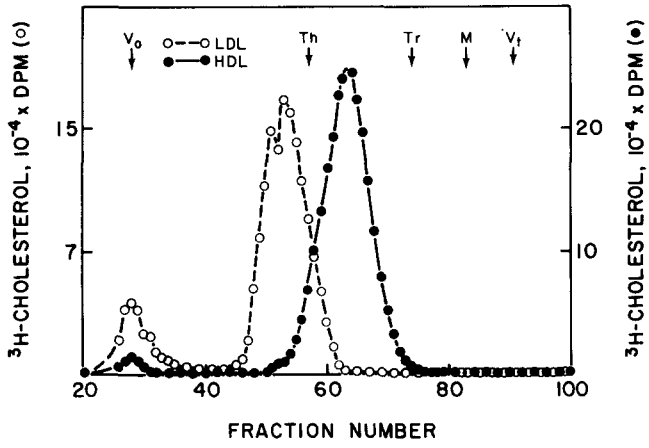

Fig. 1. Filtration of rat plasma low and high density lipoproteins on Agarose A-5m; in vitro labeling with [24,25-^{3}H]cholesterol. O - - - O, LDL; ● - - - ●, HDL; Th, thyroglobulin; Tr, transferrin; M, myoglobulin; V_t, total column volume, [^{14}C]urea marker; V_0, void volume.

1:1 containing 50.0 mg of non-radioactive cholesterol. The acetone–ethanol was removed and the specific activity of an aliquot was measured. The remainder was refluxed for 3 hr in 10% NaOH in 80% ethanol. After cooling, the alkaline solution was diluted with water and extracted with ethyl acetate. The cholesterol was recrystallized from acetone and constant specific activity was obtained after the second crystallization. The cholesterol in each sample was better than 95% pure as compared with the specific activity before saponification.

RESULTS

Labeling of the lipoproteins

Radioactive cholesterol associated with components of densities ranging from 1.006–1.063 g/ml and 1.063–1.210 g/ml after centrifugation was assumed to be bound to LDL and HDL, respectively. The putative LDL and HDL were further characterized by gel filtration on Agarose A-5m. Three radioactive peaks were observed upon gel filtration of [24,25-^{3}H]cholesterol-LDL (LDL tracer (FC)) prepared by in vitro incubation of plasma with labeled sterol followed by ultracentrifugation (**Fig. 1,** open circles). The peak present in the void volume (V_0) may represent LDL aggregates. The major portion of the radioactivity, 88%, was eluted in a broad, bifurcated peak corresponding to molecular weights (M) ranging from 1.7×10^6 to 2.1×10^6, with an average M of 1.9×10^6 and an average Stokes Radius (R_S) of ~100 Å. The double peak as well as the aggregate peak indicate some heterogeneity of the labeled LDL, possibly related to the incubation technique, since these multiple peaks were absent in the in vivo labeling procedure (see below). It should be noted that all of the [^{3}H]cholesterol radioactivity was associated with macromolecules in this as well as in the other lipoprotein preparations. Chromatography of similarly prepared [24,25-^{3}H]cholesterol-HDL (HDL tracer (FC)) (filled circles) resulted in the appearance of a single symmetrical radioactive peak, which eluted after the thyroglobulin marker and corresponded to a macromolecule with M of $\sim 4.5 \times 10^5$ and R_S of ~55 Å.

Fig. 2 shows the elution patterns of in vivo labeled [24,25-^{3}H]cholesteryl ester LDL (LDL tracer (EC)) and [24,25-^{3}H]cholesteryl ester-HDL (HDL tracer (EC)) which were obtained by ultracentrifugation of plasma taken from donor rats that had been injected with [24,25-^{3}H]cholesterol 24 hr prior to being killed. LDL radioactivity emerged in a single symmetrical peak corresponding to M of 1.9×10^6 and R_S of ~100 Å, while all of the radioactivity in HDL was associated with one peak which corresponded to M of $\sim 9.4 \times 10^5$ and R_S of ~75 Å.

The reason for the higher molecular weight of the HDL which was labeled in the donor rat is not clear. It should be noted that [^{3}H]cholesterol, which we bound to the lipoproteins by incubation, was in the free form, while the radioactivity in the LDL and HDL prepared from plasma taken from [^{3}H]cholesterol-injected rats was present in amounts greater than 90% as cholesteryl esters. In view of HDL heterogeneity, it is not unreasonable that free cholesterol and cholesteryl ester are associated with different isoproteins or that the conformation of the HDL particle is altered as the degree of esterification of cholesterol increases, causing it to appear as a larger molecule upon gel filtration. This possibility is supported by unpublished data from preliminary studies in which rats were injected with [24,25-^{3}H]cholesterol and killed 2 or 4 hr later. Labeled sterol was associated with an HDL, M of 4.5×10^5, similar to that resulting from in vitro labeling of lipoproteins. In these same studies, the

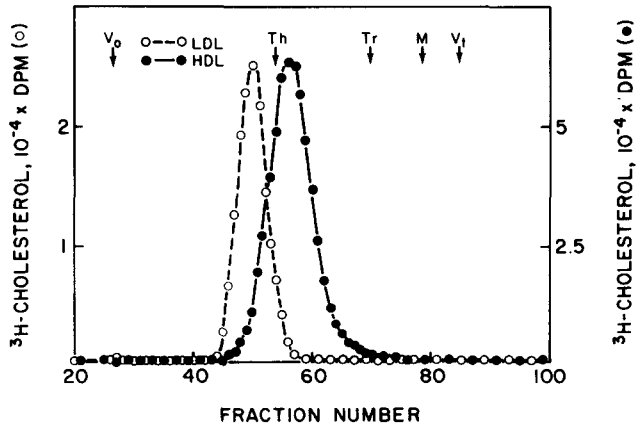

Fig. 2. Filtration of rat plasma low and high density lipoproteins on Agarose A-5m; in vivo labeling with [24,25-^{3}H]cholesterol. For legend, see Fig. 1.

radioactive LDL, where 60% of the cholesterol was free, showed a single peak whose maximum corresponded to that of the sharp, probably single component peak, M of 1.9 × 10^6, displayed by LDL labeled in vivo for 24 hr (Fig. 2), but whose broader base was reminiscent of a multicomponent fraction, possibly derived from the bifurcated peak of the in vitro labeled LDL (Fig. 1). Thus cholesterol and cholesteryl ester may be associated with different forms of LDL which do not differ sufficiently to be resolved in the gel filtration system employed.

Animal studies

Oxidation of cholesterol and cholesteryl esters bound to lipoproteins. The rates of appearance in body water of tritium oxide derived from oxidation of the labeled isopropyl group of cholesterol bound to the lipoproteins was followed for up to 60 min after administration of the tracers. **Fig. 3A** and **3B** show the oxidation of the cholesterol bound to LDL (filled circles) and HDL (open circles) by in vitro (Fig. 3A), and in vivo (Fig. 3B) techniques. The radioactivity in plasma water is expressed as % dose in plasma calculated from dpm/ml of plasma water, the weight of the rat, and the calculated plasma volume of the rat (36). It is apparent that more oxidation took place after the administration of the in vitro labeled lipoproteins as compared to the in vivo labeled material, over 10-fold for the HDL dose at 60 min. This is probably related to the fact that the radioactivity in the in vitro labeled lipoproteins was associated with free cholesterol only, which is located at the surface of the macromolecule and is presumably more available for interchange and oxidation; whereas the ^3H in the in vivo labeled HDL and LDL tracers was present in cholesteryl esters located in the lipoprotein core and probably less available for degradation (11, 16, 37, 38). It is also possible that no labeled cholesteryl esters are oxidized without prior hydrolysis and that the ^3H_2O observed in the plasma shortly after injection of the in vivo labeled lipoproteins was due to the presence of contaminating amounts of free cholesterol in the administered dose.

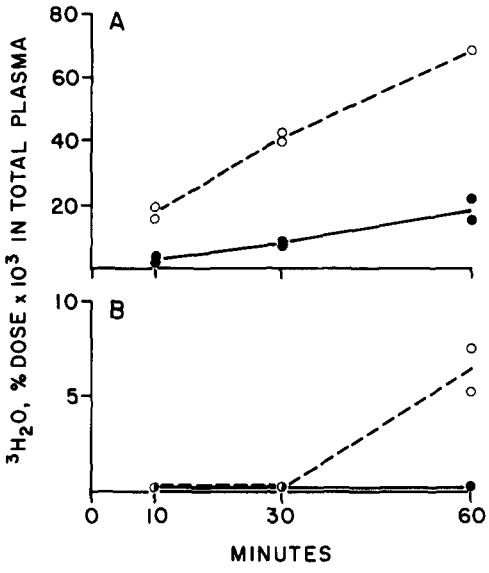

Fig. 3. First study (60 min). Plasma ^3H_2O derived from: A, [24,25-^{3}H]cholesterol-HDL and [24,25-^{3}H]cholesterol-LDL, prepared by in vitro labeling; B, [24,25-^{3}H]cholesteryl ester-HDL and [24,25-^{3}H]cholesteryl ester-LDL prepared by in vivo labeling. Each labeled species was administered to two rats and blood was taken at the times indicated and processed as described in the text. O – – – O, from HDL; ● – – – ●, from LDL.

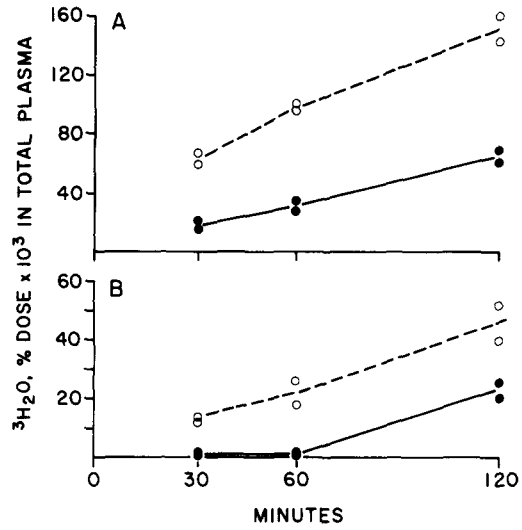

Fig. 4. Second study (120 min). Plasma ^3H_2O derived from: A, in vitro labeled lipoprotein tracers; B, in vivo labeled lipoprotein tracers. The legend is identical with Fig. 3.

Where the lipoprotein doses were obtained by in vitro labeling with [24,25-^{3}H]cholesterol (Fig. 3A), almost five times more ^3H_2O appeared after HDL tracer (FC) than after LDL tracer (FC) 60 min after administration. In Fig. 3B, the results are qualitatively similar although less ^3H_2O is produced from the in vivo labeled lipoproteins. No detectable oxidation of the labeled sterol in the LDL dose had occurred by 60 min in the recipient rats, but 60 min after the radioactive HDL dose, significant counts were measured (0.006% dose in plasma, 13 dpm/ml plasma water).

Fig. 4 shows lipoprotein cholesterol oxidation in a second study where the experimental protocol was identical with the first study, except that blood samples were taken at 30, 60, and 120 min. The results confirm the previous observations; between 30 and 120 min after injection of the labeled lipoproteins (in vitro procedure, Fig. 4A), the amount of ^3H_2O produced from [24,25-^{3}H]cholesterol-HDL ranged from 3.7–2.3 times greater than ^3H_2O derived from [24,25-^{3}H]cholesterol-LDL. When the lipoprotein tracers were labeled by in vivo procedures (Fig. 4B), radioactive HDL cholesteryl ester was preferred

TABLE 1. Plasma cholesterol radioactivity after administration of [24,25-^{3}H]cholesterol- and [24,25-^{3}H]cholesteryl ester-labeled lipoproteins (Study 1)

Time After Dose	HDL Tracer (FC)[a] % Dose in Plasma per Rat[b] FC[c]	EC	%EC[d]	LDL Tracer (FC) % Dose in Plasma per Rat FC	EC	%EC
min						
Lipoproteins labeled by in vitro method						
10	10.0	5.2	34	6.2	0.4	6.1
10	11.3	5.3	32	5.7	0.4	6.6
30	5.6	5.1	48	2.7	0.5	15
30	7.5	5.3	41	2.6	0.5	16
60	4.8	5.3	52	2.3	0.6	21
60				2.0	0.8	29
Lipoproteins labeled by in vivo method						
10	2.6	86	97	4.6	60	93
10	4.1	87	96	4.8	59	92
30	3.1	82	97	4.1	58	93
30	2.1	80	97	4.1	68	94
60	1.8	72	97	3.4	68	95
60	1.7	79	98	3.3	60	95

[a] HDL tracer (FC), [24,25-^{3}H]cholesterol-HDL; LDL tracer (FC), [24,25-^{3}H]cholesterol-LDL; HDL tracer (EC), [24,25-^{3}H]cholesteryl ester-HDL; LDL tracer (EC), [24,25-^{3}H]cholesteryl ester-LDL.

[b] Samples of plasma were collected at the indicated times. Two rats were used for each species of labeled lipoprotein. Approximately 1.5 ml of blood was taken from each rat at 10 min and 30 min, and the animals were exsanguinated at 60 min (see text).

[c] FC, free cholesterol; EC, ester cholesterol.

[d] The % radioactivity present as ester cholesterol in the administered lipoproteins was: in vitro labeled HDL, 2.5; in vitro labeled LDL, 0.2; in vivo labeled HDL, 90; in vivo labeled LDL, 91.

over labeled LDL cholesteryl ester as a precursor of ^3H_2O, about 2-fold at 120 min. Thus in each of the studies, as judged by the appearance of ^3H_2O in the plasma, the cholesterol associated with the HDL underwent loss of the terminal three carbons of the side-chain more extensively than did LDL cholesterol.

The radioactivity in plasma during the 60 min after injection of HDL tracer (FC) and LDL tracer (FC) as well as comparable data following the administration of HDL tracer (EC) and LDL tracer (EC) are shown in **Table 1.** The data from the second study, carried out for 120 min, are presented in **Table 2.** In the experiments in which [24,25-^{3}H]cholesterol was incorporated into the administered lipoprotein by the in vitro method, where the radioactivity was almost entirely associated with free cholesterol (see footnote d to Table 1 and footnote b to Table 2), less than 20% of the dose was present in the plasma between 10 and 120 min after both HDL tracer (FC) and LDL tracer (FC). In contrast, after we gave either HDL tracer (EC) or LDL tracer (EC) obtained from donor rats (in vivo technique), where the radioactivity was almost exclusively in cholesteryl esters, more than 50% of the dose remained in the plasma after 2 hr. There are at least two explanations for the differences between circulating radioactivity derived from the tracers labeled by the two methods. In the in vivo labeled lipoproteins, the [^{3}H]cholesteryl esters occupy space within the lipid core of the molecule. It is conceivable that hepatic or peripheral metabolic processes might remove the free cholesterol at the surface of the lipoprotein more rapidly than core cholesteryl esters and that such differences might be seen early after administration of the dose before esterification, hydrolysis, and ester interchange blur the observation (15, 16, 19). Alternatively or concomitantly, the free cholesterol may be more accessible to, or able to interchange with, tissue cholesterol pools, most of which are free, whereas cholesteryl esters are distributed essentially only in the plasma that contains the major cholesteryl ester pool in the animal.

Esterification of lipoprotein-bound free cholesterol in plasma. Another finding relates to the esterification of the radioactive sterol bound to the lipoproteins by the in vitro procedure. In both studies, more HDL-cholesterol underwent esterification than did comparable LDL-cholesterol; 52% vs. about 25%, respectively (Table 1), and about 65% vs. 45%, respectively (Table 2); both HDL tracer (FC) and LDL tracer (FC) contained over 96% of the label as free cholesterol.

Specific activity relationships of plasma cholesterol isolated from lipoproteins. **Table 3** shows the specific activities of cholesterol isolated from the HDL and LDL obtained from the plasma at sacrifice, 60 min after administration of the dose in study 1, and 120 min after

TABLE 2. Plasma cholesterol radioactivity after administration of [24,25-^{3}H]cholesterol- and [24,25-^{3}H]cholesteryl ester-labeled lipoprotein (Study 2)

Time After Dose	HDL Tracer (FC)[a] % Dose in Plasma per Rat[a] FC[a]	EC	%EC[b]	LDL Tracer (FC) % Dose in Plasma per Rat FC	EC	%EC
min						
Lipoproteins labeled by in vitro method						
30	6.5	8.3	56	7.9	1.8	18
30	5.5	8.0	59	6.6	1.7	20
60	4.8	9.0	65	3.9	1.7	31
60	4.0	7.7	65	3.3	1.8	35
120	4.0	6.8	63	2.9	2.2	43
120	3.1	6.4	68	2.4	2.2	48
Lipoproteins labeled by in vivo method						
30	1.8	81	98	1.2	94	99
30	1.1	72	98	0.7	82	98
60	1.2	82	99	1.6	81	98
60	1.0	62	98	0.9	56	98
120	1.3	65	98	1.1	62	98
120	1.0	56	98	1.4	49	97

[a] For nomenclature, see Table 1.

[b] The % radioactivity present as ester cholesterol in the administered lipoproteins was: in vitro labeled HDL, 3.2; in vitro labeled LDL, 0.2; in vivo labeled HDL, 91; in vivo labeled LDL, 95.

TABLE 3. Specific activity (% dose × 10^3 per μg cholesterol) of LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C) isolated from plasma obtained from rats that had been injected with lipoproteins labeled with radioactive cholesterol or radioactive cholesteryl esters

	Adminis-tered:	LDL tracer (FC)[a]		LDL tracer (EC)[b]		HDL tracer (FC)[a]		HDL tracer (EC)[b]	
	Column:	1	2	3	4	5	6	7	8
Study	Isolated:	LDL-C	HDL-C	LDL-C	HDL-C	LDL-C	HDL-C	LDL-C	HDL-C
1A 1B[c]		0.528	0.430	25.2	5.38	0.879	1.36	17.6	9.48
2A		0.595	0.748	21.5	3.90	1.03	1.60	16.0	6.67
2B		0.590	0.658	18.5	3.90	1.18	1.81	14.4	4.59

[a] For nomenclature, see footnote *a*, Table 1. Tracers were prepared by incubating plasma with [24,25-^{3}H]cholesterol and obtaining the labeled lipoproteins by ultracentrifugation. Over 90% of the [^{3}H]cholesterol was free (see text).

[b] For nomenclature, see footnote *a*, Table 1. Tracers were prepared by obtaining plasma from donor rats that had been injected with [24,25-^{3}H]cholesterol and separating the labeled lipoproteins by ultracentrifugation. Over 90% of the ^{3}H was in cholesteryl esters.

[c] In Study 1A + 1B, data for each lipoprotein pair (LDL-C and HDL-C) were obtained from two rats (total of eight rats) after combining the lipoprotein fractions from plasma obtained at sacrifice, 60 min after the dose. In the second study, 2A and 2B, we also administered each tracer to two rats but did not combine the lipoprotein fractions from plasma obtained at sacrifice, 120 min after the dose.

the dose in study 2. It can be seen by comparing the specific activity of cholesterol within a given vertical column that the figures are quite similar; this might be expected since the rats were all of the same strain, sex, age, and weight. The higher specific activity of cholesterol isolated from lipoproteins where the label was derived from [24,25-^{3}H]cholesteryl ester-HDL or -LDL (HDL or LDL tracer (EC)) as compared to HDL- or LDL-cholesterol coming from administered lipoprotein where the ^{3}H was present in free sterol (HDL or LDL tracer (FC)) is in accord with the amounts of radioactivity in the circulation after these doses (Tables 1 and 2).

One hr (study 1) or 2 hr (study 2) after the administration of LDL labeled by [^{3}H] free cholesterol (LDL tracer (FC)), the specific activities of circulating cholesterol in the LDL and HDL fractions were comparable (Table 3, columns 1 and 2), suggesting rapid interchange of surface sterol between the two lipoproteins. However, when the same class of lipoprotein was injected with the radioactivity present in cholesteryl esters (LDL tracer (EC)), the interchange or transfer of label to HDL was slower (Table 3, columns 3 and 4) and HDL-cholesterol specific activity was only 18–21% of that of LDL-cholesterol by 2 hr.

After injection of HDL labeled with [^{3}H] free cholesterol (HDL tracer (FC)), the specific activity of LDL-cholesterol isolated from plasma obtained at killing 1 and 2 hr later was about 64% that of HDL-cholesterol obtained at the same time (Table 3, columns 5 and 6) suggesting interchange, but not as fast as that after comparable labeled LDL. The most interesting result was noted in the lipoprotein cholesterol isolated after giving HDL tracer (EC), where the labeled cholesteryl esters were presumably present in the core of the molecule (Table 3, columns 7 and 8). The specific activity of cholesterol in LDL was 86–214% *greater* than the HDL-cholesterol from plasma in both of the studies, an observation that cannot be explained solely by interchange. The data are consistent with the presence of at least two concurrently operating metabolic pathways for HDL: one in which the labeled steryl ester, either by cholesteryl ester transfer or alteration of the lipoprotein molecule, is transported, or converted to LDL; the other, the major route, in which HDL is transformed to metabolic products other than LDL, i.e., degradation in the liver and replenished by intestinal and hepatic synthesis.

DISCUSSION

In two separate studies, our data show that cholesterol bound to HDL undergoes greater side-chain oxidation than does LDL-cholesterol in the intact rat. This conclusion rests on the observation that more ^3H_2O appeared after the administration of [24,25-^{3}H]cholesterol-HDL than after identically labeled LDL. Whether [^{3}H]cholesterol-LDL is oxidized directly or whether it participates as a consequence of interchange among cholesterol moieties of HDL and hepatic membranes (39, 40) cannot be answered from these studies. Indeed, the parallel slopes of the rate of appearance of ^3H_2O derived from HDL or LDL (Fig. 4) between 60 and 120 min suggest the possibility of a common source of cholesterol oxidation after injection of either labeled lipoprotein. The HDL-cholesterol was preferentially oxidized, whether the sterol was on the lipoprotein surface in the free form (37) or present as cholesteryl esters within the lipoprotein core (11). In view of the rapid metabolic activity of lipoprotein-bound free cholesterol and the slow interchange of lipoprotein-bound cholesteryl esters (16, 25, 41, 42), a faster rate of appearance and greater amounts of ^3H_2O derived from the side-chain oxidation of free sterol would be expected. This is in agreement with the utilization of free cholesterol over esterified cholesterol in bile acid formation (16, 24). Since the only way that

quantitatively significant amounts of cholesterol can be degraded in vivo is by the formation of bile acids, it follows that HDL-cholesterol appears to be a more efficient precursor of bile acids in the rat than LDL-cholesterol. It should be pointed out however that similar results might be obtained if structural alterations in the lipoprotein took place during preparation of the labeled material resulting in a less stable particle. However, we do not think this likely in view of the mild conditions of labeling the lipoproteins.

Because of the phagocytotic action of the reticuloendothelial cells of the liver on unbound cholesterol in suspension (43, 44), it was important to demonstrate that the labeled cholesterol and cholesteryl esters of the administered doses were completely bound to lipoproteins. In both Fig. 1, where the label was in free cholesterol, and Fig. 2, where cholesteryl esters were radioactive, there was no trace of ^{3}H in column eluates corresponding to the fractions in which unbound sterol or steryl esters would emerge, i.e., at V_t, the total elution volume of the column, marked by [^{14}C]urea, or beyond fraction 85.

A further criterion of purity of the labeled sterol was necessary. It had already been established that the tritium was at C-24 or distal to it (23) but radiochemical purity of the cholesterol (free and esterified) had to be demonstrated after carrying out the several steps involved in binding the cholesterol to the plasma lipoproteins and fractionating the lipoproteins into their density classes, since it is well known that incorporation of cholesterol into doses, diets, or similar preparations can be accompanied by some decomposition of the sterol (45, 46). We found that there was no change in the purity of the [24,25-^{3}H]cholesterol before and after binding to the HDL or LDL.

During the metabolism of lipoprotein-bound labeled cholesterol where the radioactivity was essentially all in free sterol (in vitro preparation), our observation that HDL-cholesterol became esterified to a greater extent than LDL-cholesterol over the period of study (Tables 1 and 2), was in accord with the widely held concept that HDL-cholesterol is the preferred substrate for esterification (47) where apoA-I functions as the activator for the LCAT enzyme (48, 49). This observation was dependent upon the fact that the administered lipoprotein species contained almost exclusively either free or esterified cholesterol. On the other hand, as judged from the behavior of lipoproteins labeled with [^{3}H]cholesteryl esters, hydrolysis of cholesteryl esters took place to a much more limited extent over the same time period.

The metabolism of HDL tracer (EC) observed in these studies (Table 3, columns 7 and 8) is consonant with a role for HDL in the disposition of cholesterol. In order to have the specific activity of cholesterol isolated from LDL exceed that of cholesterol isolated from HDL where the HDL was initially labeled, a precursor-product relation must exist between the cholesteryl ester moieties of HDL and LDL, respectively, and the rate of interchange must be relatively slow (41, 50). That the reverse reaction, the transport of cholesteryl ester from the LDL fraction to HDL, is slow can be seen from columns 3 and 4 of Table 3, where by 120 min (study 2) the specific activity of cholesterol in HDL is still only about 20% that in LDL. It has been reported that, in the rat, cholesteryl ester interchange among lipoproteins is slow as compared with humans (50–52) because of the absence of a cholesteryl ester transfer protein (53, 54). Moreover, a major portion of the HDL must be metabolized by a pathway not involving LDL and replenished from non-radioactive sources. We have shown in the present study that radioactive cholesterol and cholesteryl esters present in HDL undergo more rapid oxidation of the side-chain than do their counterparts in LDL. Previous workers have demonstrated that HDL-cholesterol is more readily converted to bile acids than is LDL-cholesterol (16, 19), and that HDL is rapidly degraded in the liver (11–14). HDL is replaced by intestinal and hepatic synthesis (55, 56), appearing first in a pre-HDL discoidal form and transforming to a spherical conformation as it takes up cholesterol from peripheral tissue sites (11, 57). Whether HDL itself transports cholesterol to the liver or transfers its cholesterol to another species of lipoprotein for disposition is not known (55).

The preferential utilization of HDL-cholesterol for bile acid formation is another example of the greater metabolic activity of cholesterol associated with this species of lipoprotein which is also more readily converted to steroid hormones in the adrenal glands and gonads of the rat (58–60).

Labeling specific lipoproteins with free or esterified [24,25-^{3}H]cholesterol may afford novel and useful probes to evaluate the contribution of individual lipoprotein species to cholesterol oxidation (bile acid formation) in normal and pathological conditions as well as in isolated cell fractions, cell cultures, and perfusion systems.

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