

In vitro incorporation of radiolabeled cholesteryl esters into high and low density lipoproteins

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Abstract We have developed and validated a method for in vitro incorporation of radiolabeled cholesteryl esters into low density (LDL) and high density lipoproteins (HDL). Radiolabeled cholesteryl esters dissolved in absolute ethanol were mixed with LDL or HDL in the presence of lipoprotein-deficient serum (LPDS) as a source of core lipid transfer activity. The efficiency of incorporation was dependent on: *a*) the core lipid transfer activity and quantity of LPDS, *b*) the mass of added radiolabeled cholesteryl esters, *c*) the length of incubation, and *d*) the amount of acceptor lipoprotein cholesterol. The tracer incorporation was documented by repeat density gradient ultracentrifugation, agarose gel electrophoresis, and precipitation with heparin-MnCl₂. The radiolabeling conditions did not affect the following properties of the lipoproteins: 1) chemical composition, 2) electrophoretic mobility on agarose gels, 3) hydrated density, 4) distribution of apoproteins on SDS gels, 5) plasma clearance rates, and 6) immunoprecipitability of HDL apoproteins A-I and A-II. Rat HDL containing radiolabeled cholesteryl esters incorporated in vitro had plasma disappearance rates identical to HDL radiolabeled in vivo.—Terpstra, A. H. M., R. J. Nicolosi, and P. N. Herbert. In vitro incorporation of radiolabeled cholesteryl esters into high and low density lipoproteins. *J. Lipid Res.* 1989. 30: 1663-1671.

Supplementary key words cholesterol • lipoproteins • core lipid transfer protein

The discovery that lipoprotein core lipids have metabolic properties different from the surface proteins has stimulated considerable interest in methods for radiolabeling the cholesteryl ester of individual lipoprotein classes. Cholesteryl esters and lipoproteins can be radiolabeled in vivo by administration of radioactive unesterified cholesterol. Several methods for in vitro labeling have also been described. One approach takes advantage of the lecithin:cholesterol acyltransferase (LCAT) activity of plasma to produce radiolabeled cholesteryl esters from radiolabeled free cholesterol (1, 2). Other techniques have involved transfer of radiolabeled cholesteryl ester coated on glass tubes (3), transfer from lipid emulsions (4, 5), partial delipidation of the lipoproteins and reconstitution with labeled cholesteryl ester (6, 7), and adding labeled cholesteryl ester to lipoprotein solutions (8, 9).

In vivo and in vitro methods employing LCAT introduce radiolabeled free cholesterol as well as cholesteryl esters into the lipoproteins. This problem can be avoided by using radiolabeled cholesteryl esters but the efficiency of cholesteryl ester incorporation has generally been low (3-9). In the present study we describe an efficient procedure for labeling the lipoproteins exclusively in the cholesteryl ester moiety. Possible effects of the labeling procedure on the properties of the lipoproteins were studied and plasma clearance rates of lipoproteins labeled in vitro were compared with those radiolabeled in vivo.

MATERIALS AND METHODS

Preparation of lipoproteins and lipoprotein-deficient serum (LPDS)

Benzamidine and aprotinin, at final concentrations of 20 mmol and 234,000 kallikrein inhibitory units per liter (10), were added to blood anticoagulated with Na₂-EDTA. Human LDL and HDL were prepared by sequential ultracentrifugation (11). Rat lipoproteins were isolated by a combination of differential and density gradient ultracentrifugation as previously described (12). Lipoprotein-deficient serum (LPDS) ($d > 1.21$ g/ml) and lipoproteins were dialyzed against 0.15 mol/l NaCl and 0.3 mmol/l sodium ethylmercurithiosalicylate (thiomersal) (11) and kept under nitrogen during dialysis and storage. Purity of the isolated lipoproteins was assessed by agarose electrophoresis (13). LCAT in LPDS was inactivated by heating in a waterbath at 60°C for 10 min (14, 15).

Abbreviations: VLDL, very low density lipoproteins ($d < 1.006$ g/ml); LDL, low density lipoproteins ($1.019 < d < 1.063$ g/ml); HDL, high density lipoproteins ($1.063 < d < 1.21$ g/ml), subdivided into HDL₂ ($1.063 < d < 1.10$ g/ml) and HDL₃ ($1.10 < d < 1.21$ g/ml); LCAT, lecithin:cholesterol acyltransferase; SDS, sodium dodecyl sulfate; LPDS, lipoprotein-deficient serum ($d < 1.21$ g/ml); FCR, fractional catabolic rate (h^{-1}).

Radioisotopes

Cholesteryl [9,10-³H]oleate (sp act 107 TBq/mol), cholesteryl [1-¹⁴C]oleate (sp act 2.09 TBq/mol), [1,2,6,7-³H(N)]cholesteryl oleate (sp act 2.95 PBq/mol), [4-¹⁴C]cholesteryl oleate (sp act 2.20 TBq/mol), and [1,2-³H(N)]cholesterol (sp act 1.72 PBq/mol) were purchased from New England Nuclear (Boston, MA 02118). The purity of these compounds was verified by thin-layer chromatography (16) and more than 97% of all radioactive cholesteryl ester preparations comigrated with a cholesteryl ester standard. [1,2-³H(N)]cholesterol was purified by preparative thin-layer chromatography and eluted from the silica gel with chloroform-methanol 9:1 (v/v). After purification, more than 98% of the tracer comigrated with a cholesteryl ester standard. ¹²⁵I and ¹³¹I were also purchased from New England Nuclear.

Animals

Sprague-Dawley (250–480 g) rats used as lipoprotein donors for the studies comparing *in vivo* and *in vitro* labeled HDL were fed a semipurified diet (Teklad, Madison, WI 53711) containing 20% casein, 50% sucrose, 16% corn starch, 5% corn oil, 5% cellulose, and 4% vitamin and mineral mixtures (w/w). This diet reduces the fraction of polyunsaturated cholesteryl esters (17) which might be liable to oxidation during storage and incubation. All other donor rats and rats used for lipoprotein kinetic studies were fed a chow diet (Ralston Purina, St. Louis, MO 63164).

Preparation of cholesteryl ester radiolabeled lipoproteins *in vitro*

Isolated lipoproteins and LPDS were mixed in 0.15 M NaCl containing thiomerosal (0.3 mmol/l (11)), aprotinin (234,000 kallikrein inhibitory units per liter (12)), and glutathione (0.65 mmol/l (18)) at a final volume of 4 ml. Radiolabeled cholesteryl oleate dissolved in benzene was transferred to a 4-ml polypropylene conical tube and evaporated to dryness under a stream of nitrogen. The tracer was resolubilized in 50 μ l absolute ethanol, incubated for 5 min at 37°C, vortexed intermittently, and then added dropwise to a vortexing solution of lipoproteins and LPDS. The mixture was incubated for 24 h at 37°C in a shaking water bath under nitrogen, and the radiolabeled lipoproteins were isolated by density gradient ultracentrifugation (12).

Preparation of rat HDL labeled with cholesteryl esters *in vivo*

Twenty-eight MBq [1,2-³H(N)]cholesterol dissolved in 50 μ l absolute ethanol was added to 2 ml rat plasma and administered intravenously to a rat. Plasma was collected after 24 h, infused into a second rat, and harvested 1 h later. Biological screening reduced the fraction of tracer in

unesterified cholesterol from 27 to 6%. HDL isolated after density gradient ultracentrifugation contained 70 kBq of [1,2-³H(N)]cholesteryl esters.

Preparation and incubation of radioiodinated lipoproteins

LDL and HDL were radioiodinated with ¹²⁵I and ¹³¹I using a modification (19) of the iodine monochloride technique (20). The radioiodinated lipoproteins were separately added to aliquots of cynomolgus LPDS (120 mg protein) and unlabeled lipoproteins (5 μ mol cholesterol). Thiomerosal, benzamidine, and glutathione were added and the volume was adjusted to 4 ml with 0.15 M NaCl. Samples containing the ¹²⁵I-labeled LDL and HDL were mixed with 50 μ l ethanol and incubated at 37°C for 24 h to simulate conditions of cholesteryl ester labeling. Ethanol was not added to control samples which were kept at 4°C for the same period.

Cholesteryl ester transfer studies

A tracer amount of radiolabeled LDL or HDL was added to an incubation mixture containing human LDL (6 μ mol cholesteryl ester when the transfer of radiolabeled LDL preparations was studied and 18 μ mol cholesteryl ester in studies with radiolabeled HDL), HDL (6 μ mol cholesteryl ester), and heat-inactivated LPDS (180 mg) in a total volume of 3.5 ml. These concentrations are only roughly comparable to those in human plasma, but they were chosen to promote a relatively high rate of flux of cholesteryl esters between LDL and HDL. The mixture was incubated at 37°C in a shaking waterbath for 5 h; timed aliquots were taken, and LDL were precipitated with a heparin-MnCl₂ solution (21). Tracer remaining in the supernatant was radioassayed and the apparent bidirectional flux of cholesteryl ester between the LDL and HDL was calculated using a closed two-pool model as described by Shipley and Clark (22) and adapted by Barter and Jones (23). At *t*₀, 98–100% of labeled LDL was precipitable, and 84–97% of labeled HDL remained in the supernatant fraction.

Lipoprotein turnover studies

Rats were anesthetized with pentobarbital (5 mg/100 g body weight) the night before tracer injection, and a silastic catheter (i.d. 0.51 mm, o.d. 0.94 mm, Dow Corning Corporation, Midland, MI 48640) was surgically implanted in the right jugular vein and advanced into the atrium. The catheters were externalized and kept open by a constant infusion of saline at a rate of 0.3 ml/h. Eight to 12 blood samples (0.3–0.6 ml) were taken over a period of 30–35 h after tracer administration. Radioactivity was expressed as a fraction of that in plasma at 10 min after injection. Fractional catabolic rates (FCR) were calculated as the reciprocal of the area under the plasma decay curves (22).

Analytical methods

Cholesterol (24) and triglycerides (25) were measured enzymatically on a Gilford Impact 400 Analyzer with reagents supplied by Worthington, Inc. (Freehold, NJ 07728). Phospholipids were assayed as previously described (26). Lipoprotein protein was estimated using a modification (27) of the method of Lowry et al. (28) and total protein in the LPDS was determined by the biuret technique (29). Lipoprotein electrophoretograms (13) in 1% agarose (Corning Universal, Electrophoresis Film^R) were stained with Fat Red 7B Stain (Fisher Corning Scientific, Pittsburgh, PA 15219). The apoproteins of LDL and HDL were separated by electrophoresis in SDS polyacrylamide gels (30), 3% monomer concentration for LDL and 7.5% for HDL apoproteins. Free and esterified cholesterol were separated on plastic thin-layer chromatography plates (Eastman Kodak, Rochester, NY 14650) developed in hexane-ethanol-acetic acid 80:25:2 (v/v) (16). After lipids were visualized with iodine vapor, the sheets were cut and assayed for radioactivity. ¹⁴C and ³H radioactivities were measured in a Packard Tricarb 4530 scintillation counter with a dual-label quench-correcting program. Instagel (Packard Instrument Company, Downers Grove, IL 60515) was used as scintillation liquid. ¹²⁵I

TABLE 1. Dependence of apparent cholesteryl ester flux between LDL and HDL on the source of LPDS^a

Source of Lipoprotein-Deficient Serum	Apparent Cholesteryl Ester Flux nmol/h
Rat (<i>Rattus norvegicus</i>)	2
Human (<i>Homo sapiens</i>)	9
Tamarin (<i>Saguinus oedipus</i>) ^b	10
Formosan macaque (<i>Macaca cyclopis</i>) ^c	14
Common marmoset (<i>Callithrix jacchus</i>) ^b	15
Rabbit (<i>Oryctolagus cuniculus domesticus</i>)	15
Capuchin (<i>Cebus albifrons</i>) ^b	19
Squirrel monkey (<i>Saimiri sciureus</i>) ^b	20
Olive baboon (<i>Papio anubis</i>) ^c	20
Rhesus monkey (<i>Macaca mulatta</i>) ^c	24
Owl monkey (<i>Aotus trivirgatus</i>) ^b	31
Cynomolgus monkey (<i>Macaca fascicularis</i>) ^c	43
Spider monkey (<i>Ateles geoffroyi</i>) ^b	46
Tamarin (<i>Saguinus fuscicollis</i>) ^b	55

^aLipoprotein-deficient serum (6 mg protein), human LDL (296 nmol cholesteryl ester), human HDL (246 nmol cholesteryl ester), and a tracer amount of cholesteryl [1-¹⁴C]oleate-labeled LDL were incubated for 110 min at 37°C. The total volume of the incubation mixture was 530 μ l. After incubation, the LDL were precipitated with a heparin-MnCl₂ solution using human whole serum as carrier, and the apparent cholesteryl ester flux between LDL and HDL was calculated (22, 23). The amounts of LPDS, LDL, and HDL were chosen arbitrarily, and 99.6% of the tracer in the LDL preparation was precipitable with heparin-MnCl₂. The percentage of tracer transfer during the 110-min incubation ranged from 5% for human LPDS to 24% for tamarin LPDS.

^bNew World monkey.

^cOld World monkey.

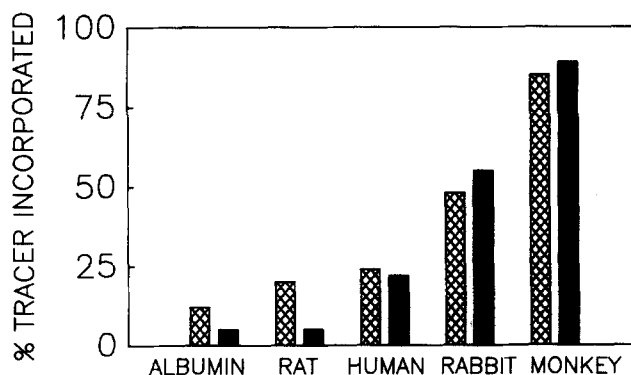


Fig. 1. Effect of the source of LPDS on the efficiency of incorporation of radiolabeled cholesteryl esters into human LDL (hatched bars) and HDL (solid bars). The incubation mixture (4 ml) contained 120 mg LPDS or bovine serum albumin, 5 μ mol lipoprotein cholesterol, and 75 kBq cholesteryl [1-¹⁴C]oleate (36 nmol). Samples were incubated at 37°C for 24 h.

and ¹³¹I radioactivities were determined simultaneously with a Packard Multi-Prias dual-channel gamma ray spectrometer.

RESULTS

Efficiency of tracer incorporation into lipoproteins

Pilot studies using human LPDS showed little incorporation of cholesteryl [1-¹⁴C]oleate into lipoproteins. We postulated that this might be related to core lipid transfer activity; this was examined in a number of species (Table 1). Little activity was found in rat LPDS and highest activities were found in two New World monkey species, the tamarin and spider monkeys, and in the Old World cynomolgus monkey. Other closely related New and Old World species had activities almost as low as in humans (Table 1).

Cholesteryl [1-¹⁴C]oleate incorporation into lipoproteins was next examined using rat, human, rabbit, and monkey LPDS. The efficiency of tracer incorporation was greatest with cynomolgus LPDS, least with rat, and intermediate with rabbit and human LPDS (Fig. 1). The rank-order of incorporation efficiency was the same as the rank-order of core lipid transfer activity (Table 1).

Since human LPDS is available in quantity, it was used to further define conditions for optimal cholesteryl ester incorporation. Greater masses of tracer added to fixed amounts of lipoprotein and LPDS resulted in lower incorporation efficiencies, particularly into HDL (Fig. 2A). Conversely, using tritiated tracer of higher specific activity resulted in very efficient incorporation with comparable results using human, rabbit, and monkey LPDS (Fig. 3). Increasing the mass of acceptor lipoprotein did not improve efficiency and, in the case of HDL, gave even less incorporation (Fig. 2B). Longer periods of incubation

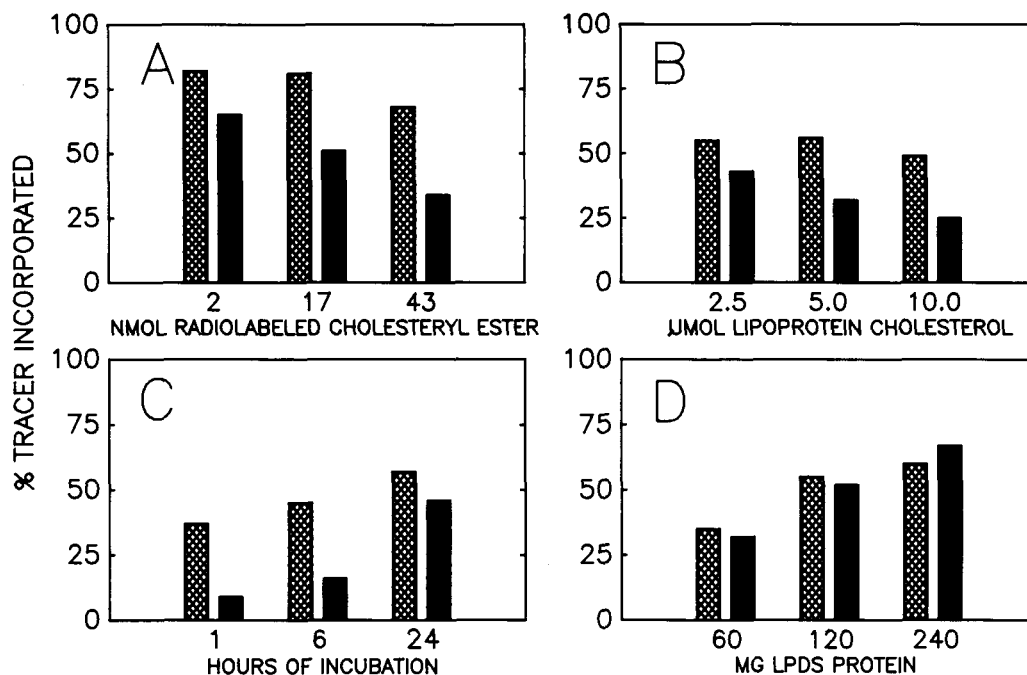


Fig. 2. Variation of cholesteryl ester incorporation into human LDL (hatched bars) and HDL (solid bars) with mass of radiolabeled tracer (panel A), mass of acceptor lipoprotein (panel B), length of incubation (panel C), and quantity of LPDS (panel D). Incubation mixtures (4 ml) contained: panel A, 120 mg human LPDS and 5 μ mol lipoprotein cholesterol; panel B, 120 mg LPDS and 75 kBq cholesteryl [$1\text{-}^{14}\text{C}$]oleate; panel C, 120 mg LPDS, 5 μ mol lipoprotein cholesterol, and 56 kBq cholesteryl [$1\text{-}^{14}\text{C}$]oleate; and panel D, 5 μ mol lipoprotein cholesterol and 75 kBq (LDL-study) or 44 kBq (HDL-study) cholesteryl [$1\text{-}^{14}\text{C}$]oleate. Incubations were for 24 h at 37°C.

resulted in better incorporation into both LDL and HDL (Fig. 2C) and greater amounts of LPDS (Fig. 2D) also improved tracer incorporation.

Assessment of tracer cholesteryl ester incorporation

Physical properties. Ultracentrifugation, heparin- MnCl_2 precipitation, and electrophoresis were used to assess the integrity of lipoproteins containing cholesteryl [$1\text{-}^{14}\text{C}$]o-

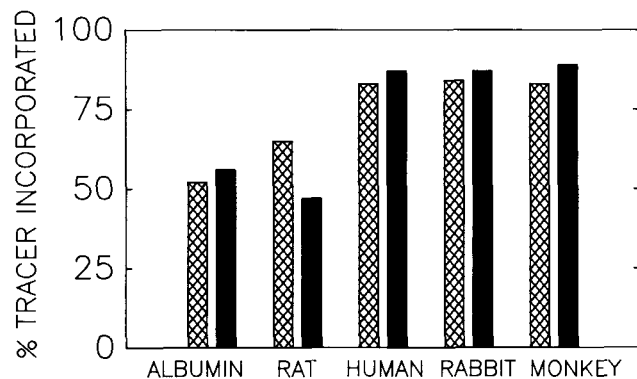


Fig. 3. Efficiency of incorporation of tritiated cholesteryl oleate into LDL (hatched bars) and HDL (solid bars) using bovine serum albumin and LPDS from rats, humans, rabbits, and monkeys. The incubation mixtures contained 125 mg LPDS or albumin, 5 μ mol lipoprotein cholesterol, and 75 kBq (0.7 nmol) of cholesteryl [$9,10\text{-}^3\text{H}$]oleate and were incubated 24 h at 37°C.

leate. Human LDL and HDL, labeled in the presence of cynomolgus and rat LPDS, were reisolated and subjected to ultracentrifugation once again. More than 90% of the labeled lipoproteins was recovered in the expected density ranges. Moreover, 99% of tracer in LDL was precipitable with heparin- MnCl_2 whereas 90–95% of HDL tracer was not precipitable. The bulk of the two tracers (90–98%) migrated together with the respective lipoproteins on 1% agarose gels.

Cholesteryl ester transfer studies. Cholesteryl esters are located almost exclusively in the core of native lipoproteins and do not readily exchange between lipoproteins in the absence of core lipid transfer activity (31). We used capacity of tracer to exchange between LDL and HDL as a measure of the goodness of tracer incorporation into these lipoproteins. These experiments required use of both ^3H and ^{14}C cholesteryl esters which behave identically in the transfer reaction (Table 2).

First, we compared rat HDL cholesteryl esters labeled with ^{14}C in vitro using cynomolgus LPDS and rat HDL labeled in vivo after injection of ^3H cholesterol. The flux of in vitro-labeled [^{14}C]HDL tracer between HDL and LDL was 1.38-fold greater than [^3H]HDL labeled in vivo. This observation suggested that HDL labeled in vitro had an increased exchange capacity. However, the in vitro-labeled [^{14}C]HDL contained only radiolabeled cholesteryl oleate, whereas [^3H]HDL labeled in vivo contained a

TABLE 2. Dependence of apparent cholesteryl ester flux between LDL and HDL on the protein or source of LPDS used for tracer incorporation

Labeled Donor Lipoproteins	Source of LPDS or Protein Used to Label Donor Lipoproteins	Isotope Used to Trace Cholesteryl Ester	Apparent Cholesteryl Ester Flux <i>nmol/h</i>	Ratio of Fluxes ($^{14}\text{C}/^3\text{H}$)
LDL	Monkey	^{14}C	215	1.00
	Monkey	^3H	215	
LDL	Monkey	^{14}C	194	1.01
	Human	^3H	194	
LDL	Monkey	^{14}C	197	0.64
	Rat	^3H	309	
LDL	Monkey	^{14}C	196	0.48
	Albumin	^3H	406	
HDL	Monkey	^{14}C	533	1.00
	Monkey	^3H	523	
HDL	Monkey	^{14}C	469	0.97
	Human	^3H	486	
HDL	Monkey	^{14}C	442	0.99
	Rat	^3H	447	
HDL	Monkey	^{14}C	435	1.05
	Albumin	^3H	415	

*Human lipoproteins were labeled with radioactive cholesteryl esters during a 24-h incubation in the presence of albumin or LPDS from various sources. The labeled lipoproteins were reisolated. Exchange of tracer between LDL and HDL was then examined under incubation conditions described in Methods. Apparent cholesteryl ester flux between LDL and HDL was calculated as described (22, 23).

mixture of several fatty acids. The fatty acyl chain of cholesteryl esters is known to affect transfer rates (32). This was shown for our preparations by exchanging cholesteryl esters from the in vivo- and in vitro-labeled rat HDL to human LDL. Back exchange of the [^3H]LDL and [^{14}C]LDL to human HDL was then compared and the flux of the ^{14}C tracer was again 1.42-fold higher than that of the ^3H tracer. Thus, differences in exchangeability of in vitro- and in vivo-labeled HDL cholesteryl esters appear due to fatty acid content rather than artifacts of incorporation.

In subsequent experiments, we assumed that cholesteryl [$1\text{-}^{14}\text{C}$]oleate, introduced into lipoproteins in the presence of cynomolgus LPDS, was comparable to native lipoprotein cholesteryl esters. We compared the activity of these reference lipoproteins in the core lipid transfer reaction with lipoproteins containing cholesteryl [$9,10\text{-}^3\text{H}$]oleate introduced using other species of LPDS or albumin (Table 2).

Tracer introduced into LDL and HDL in the presence of human LPDS behaved identically to tracer introduced in the presence of cynomolgus monkey LPDS; and this was also true for tracer incorporated into HDL in the presence of rat LPDS and bovine serum albumin (Table

2). However, tracer introduced into LDL in the presence of rat LPDS or bovine serum albumin had a higher transfer rate (Table 2). Moreover, the cholesteryl ester transfer rates of these LDL were not reproducible and electrophoresis on 1% agarose gels showed variable (75–90%) comigration of tracer with LDL.

Kinetic properties of labeled lipoproteins. These experiments required use of ^3H - and ^{14}C -labeled cholesteryl esters in dual-label experiments. Therefore, we first examined whether ^3H - and ^{14}C -radiolabeled cholesteryl esters had the same in vivo kinetic properties when incorporated into HDL. Rat HDL were labeled in the presence of cynomolgus monkey LPDS with [$1,2,6,7\text{-}^3\text{H}(\text{N})$]cholesteryl oleate and [$4\text{-}^{14}\text{C}$]cholesteryl oleate. The radiolabeled lipoprotein preparations were mixed together, administered to six rats, and the pattern of plasma decay was determined. The time course of clearance of the two tracers was indistinguishable (Fig. 4A) and the ratio of the $^3\text{H}/^{14}\text{C}$ in the 10-min samples was identical to that in the sample infused (Table 3).

Next, we compared the kinetic properties of rat HDL labeled with cholesteryl esters in vitro and in vivo. In vitro labeling was accomplished with [$4\text{-}^{14}\text{C}$]cholesteryl oleate using cynomolgus LPDS and a 24-h incubation. HDL were labeled in vivo by administering unesterified [$1,2\text{-}^3\text{H}(\text{N})$]cholesterol and harvesting the HDL 24 h later. The two tracers had almost identical disappearance curves (Fig. 4B) and calculated FCRs from rat plasma (Table 3).

Further, we compared the biological properties of rat HDL labeled with cholesteryl esters in the presence of various sources of LPDS. Rat HDL were labeled with [$1,2,6,7\text{-}^3\text{H}(\text{N})$]cholesteryl oleate in the presence of albumin, human LPDS, or rat LPDS, and with [$4\text{-}^{14}\text{C}$]cholesteryl oleate in the presence of cynomolgus monkey LPDS. All incorporations were accomplished using a 24-h incubation. The plasma disappearance rate of the HDL labeled in the presence of human LPDS was slightly lower (about 5%) (Fig. 4C, Table 3), whereas HDL labeled in the presence of rat LPDS had a plasma disappearance rate similar to HDL labeled with cynomolgus LPDS (Fig. 4D, Table 3). HDL labeled in the presence of albumin, in contrast, had a higher plasma disappearance rate than HDL labeled with cynomolgus LPDS (Fig. 4E, Table 2).

Effect of prolonged incubation on lipoprotein properties

Human LDL and HDL were incubated at 4°C and 37°C for 24 h in the presence of cynomolgus LPDS. Mixtures incubated at 37°C contained 1.25% (v/v) ethanol whereas those at 4°C did not. Prolonged incubation did not alter the chemical composition (percent dry weight of protein, phospholipid, cholesterol, and triglyceride), electrophoretic mobility, or hydrated density of LDL on den-

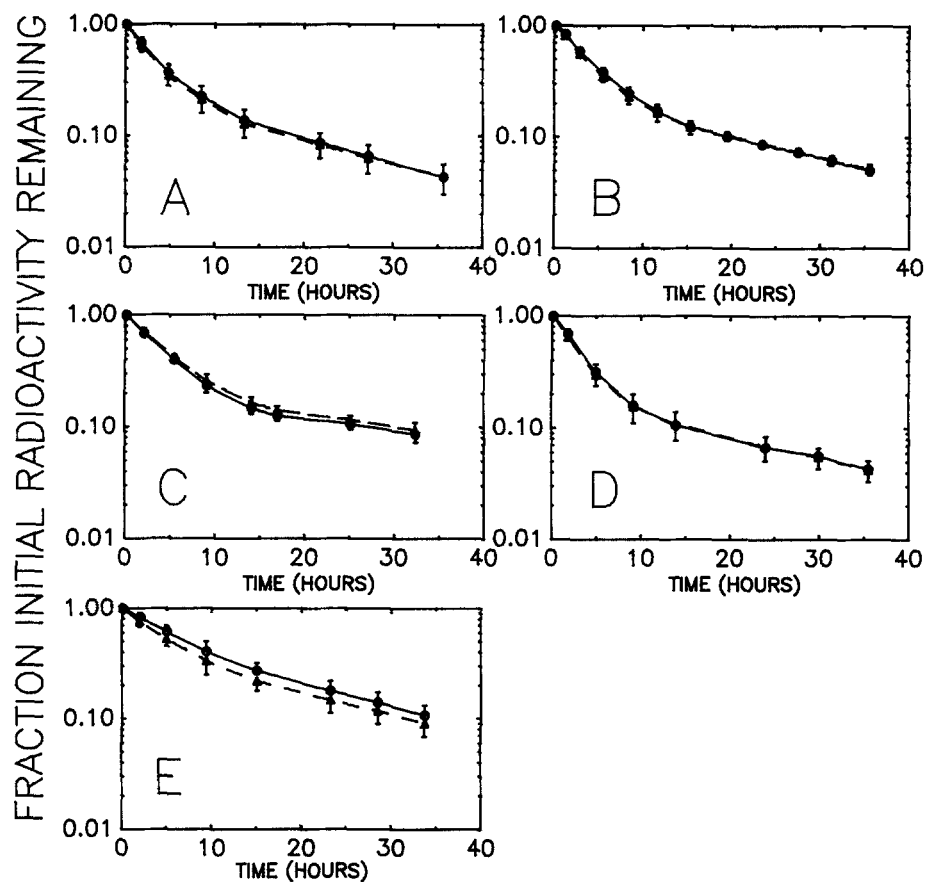


Fig. 4. Rat plasma disappearance curves of HDL labeled with cholesteryl esters under different conditions. In each experiment, the clearance rate of rat HDL radiolabeled in vitro with $[4\text{-}^{14}\text{C}]$ cholesteryl oleate ($\circ\text{---}\circ$) using cynomolgus LPDS is compared with: panel A, HDL radiolabeled in vivo with $[1,2\text{-}^3\text{H(N)}]$ cholesteryl esters ($\triangle\text{---}\triangle$); and panels B-E, HDL labeled in vitro with $[1,2,6,7\text{-}^3\text{H(N)}]$ cholesteryl oleate ($\triangle\text{---}\triangle$) using cynomolgus LPDS (panel B), human LPDS (panel C), rat LPDS (panel D), and albumin (panel E). Each value represents the mean \pm SD of six rats.

TABLE 3. Fractional catabolic rates of rat HDL radiolabeled with cholesteryl esters under various conditions^a

Source of LPDS or Protein Used to Label Lipoproteins	Isotope Used to Trace Cholesteryl Esters	Dose Administered			Ratio $^3\text{H}/^{14}\text{C}$	
		kBq	Total Cholesterol	FCR	Infusion Sample	10-Min Sample
Monkey ^b	^{14}C	39.62 ± 2.81	1.16 ± 0.09	0.138 ± 0.023	2.34	2.32 ± 0.02
Monkey	^3H	92.61 ± 6.70		0.139 ± 0.023		
Monkey In vivo	^{14}C	11.54 ± 1.85	1.49 ± 0.23	0.134 ± 0.013	0.92	0.92 ± 0.01
	^3H	10.58 ± 1.59		0.135 ± 0.016		
Monkey Human	^{14}C	19.61 ± 0.90	3.72 ± 0.17	0.102 ± 0.021	2.20	2.18 ± 0.02
	^3H	43.03 ± 1.97		$0.097 \pm 0.022^*$		
Monkey	^{14}C	37.59 ± 2.37	1.08 ± 0.07	0.163 ± 0.031	1.16	1.10 ± 0.01
Rat	^3H	43.49 ± 2.74		0.167 ± 0.034		
Monkey	^{14}C	44.47 ± 1.96	1.98 ± 0.04	0.080 ± 0.015	1.27	1.23 ± 0.01
Albumin ^c	^3H	56.76 ± 2.48		$0.092 \pm 0.018^{**}$		

^aData are expressed as mean \pm SD of six rats. FCRs within each experiment were statistically analyzed using a paired, two-tailed *t*-test; * $P < 0.01$; ** $P < 0.001$. Male rats were used in the first three experiments and female rats in the last two.

^bCynomolgus monkey.

^cBovine serum albumin.

sity gradient ultracentrifugation. Incubated and control LDL had similar radioactivity distributions on 3% SDS gels (Fig. 5A). Moreover, the FCRs of incubated and control LDL preparations did not differ (0.075 ± 0.004 and $0.074 \pm 0.004 \text{ h}^{-1}$; mean \pm SD of six rats) and the ratio of $^{125}\text{I}/^{131}\text{I}$ in the 10-min samples (1.03) was similar to that in the sample infused (1.05).

Incubation also did not alter HDL chemical composition or mobility on agarose gels. However, the usually clear separation between HDL₂ and HDL₃ seen on density gradient ultracentrifugation was lost after a 24-h incubation; and a small increase (3%) in the HDL₂ fraction occurred at the expense of the HDL₃. These changes in HDL subfractions did not occur when incubations were limited to 6 h. Incubated and control preparations of radioiodinated HDL had similar radioactivity distributions on 7.5% SDS gels (Fig. 5B) and similar clearance rates from rat plasma (both $0.102 \pm 0.008 \text{ h}^{-1}$, mean \pm SD of five animals). Finally, we examined the immunoprecipitability of radioiodinated apoA-I and apoA-II. The fraction of radioactivity precipitable with anti-apoA-I was 52% in the incubated and 53% in the control HDL. The respective values with an antiserum to apoA-II were 35% and 38%.

DISCUSSION

Studies described in this report validate a convenient method for labeling lipoproteins with defined cholesteryl esters. We observed that the core lipid transfer activity of the LPDS used to facilitate incorporation of cholesteryl esters affected both the efficiency of incorporation and the biologic properties of the tracer lipoproteins. The extent of incorporation correlated directly with the LPDS core lipid transfer activity (Table 1 and Fig. 1). However, core lipid transfer protein was not absolutely essential since

some incorporation was observed even with albumin and rat LPDS (Fig. 1). Cholesteryl esters introduced into HDL using the latter protein sources had apparently normal capacity to participate in the core lipid transfer reaction (Table 2). HDL cholesteryl esters labeled using rat LPDS also had plasma FCRs similar to HDL labeled with cynomolgus LPDS; but cholesteryl esters incorporated with albumin were cleared faster from plasma (Table 3). Neither albumin nor rat LPDS induced satisfactory tracer incorporation into LDL. LDL tracers produced with these proteins had erratic electrophoretic properties and high exchange rates in the core lipid transfer reaction (Table 2).

Tracer incorporation was also facilitated by longer in vitro incubation times and larger quantities of LPDS. Conversely, increasing either the mass of acceptor lipoproteins or the mass of tracer reduced the incorporation efficiency when the quantity of LPDS was held constant. A greater mass of acceptor lipoprotein may simply compete with tracer for binding to the core lipid transfer protein.

The effect of tracer mass on the fraction incorporated into acceptor lipoproteins was apparent at tracer:acceptor mass ratios as low as 1:2500 (Figs. 2A and 3). We do not know if this is related to the limited amounts of core lipid transfer protein available to tracer or to the physiological state of the tracer. We assume cholesteryl esters added in alcohol to aqueous solutions either form microsuspensions or combine with lipoprotein surface lipids to form artificial lipoproteins. In any event, it is clear that tracer of high specific activity is preferable (Fig. 3) if millicurie or greater quantities of radioactive lipoproteins are needed.

The conditions of tracer incorporation involving prolonged incubation had minimal effects on the properties of lipoproteins. Precipitability with heparin-MnCl₂, hydrated density assessed by ultracentrifugation, elec-

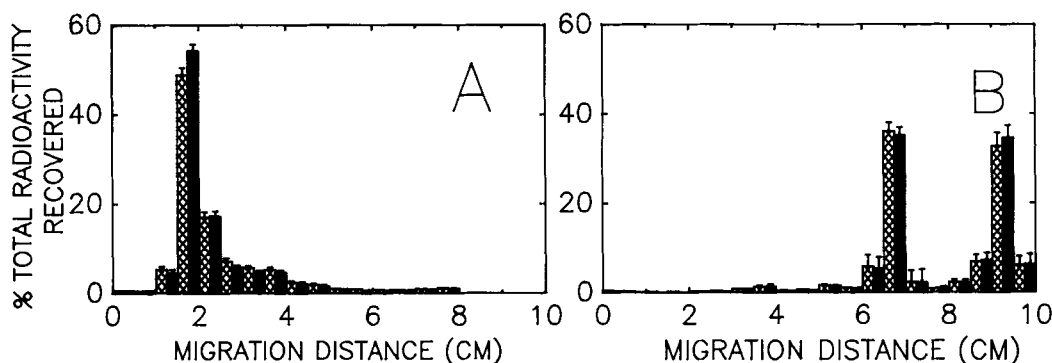


Fig. 5. Distribution of radioactivity in nonincubated (hatched bars) and incubated (solid bars) lipoproteins. The LDL (panel A) were analyzed on 3% gels ($n = 5$) and HDL (panel B) were analyzed on 7.5% gels ($n = 6$). The radioactivity was assayed in 0.5-cm gel slices. Each value represents the mean \pm SD.

trophoretic mobility, chemical composition, and apolipoprotein integrity were generally unchanged. The single exception was some loss of the separation of HDL₂ from HDL₃ after a 24-h incubation and this change was not observed after a 6-h incubation. It should be stressed that labeling conditions were strictly controlled with addition of proteolytic inhibitors, antioxidant, and incubation and dialysis in a nitrogen atmosphere.

Tracers prepared in vitro had plasma clearance curves in the rat identical to those produced in vivo (Fig. 4 and Table 3). We have not studied tracer kinetic properties in animals having plasma core lipid transfer activity. It is possible that a single species of cholesteryl ester, such as cholesteryl oleate, may not adequately trace the fate of a heterogeneous mixture of cholesteryl esters in species with core lipid transfer activity. Morton (32) has demonstrated that the core lipid transfer protein-mediated exchange of cholesteryl esters between lipoproteins is dependent on the nature of the fatty acyl chain. We have shown here that rat HDL cholesteryl esters produced in vivo indeed exchange to LDL more rapidly than HDL cholesteryl oleate introduced in vitro. Moreover, there is evidence in the monkey that different species of cholesteryl esters have different plasma turnover rates (33). It has not yet been shown that these differences are related to core lipid transfer protein-mediated exchange. ■

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