Rapid labeling of lipoproteins in plasma with radioactive cholesterol. Application for measurement of plasma cholesterol esterification

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Summary In order to efficiently and rapidly label lipoproteins in plasma with [^{3}H]cholesterol, micelles consisting of lysophosphatidylcholine (lysoPC) and [^{3}H]cholesterol (molar ratio, 50:1) were prepared. When trace amounts of these micelles were injected into plasma, [^{3}H]cholesterol rapidly equilibrated among the plasma lipoproteins, as compared to [^{3}H]cholesterol from an albumin-stabilized emulsion. The distributions of both [^{3}H]cholesterol and unlabeled free cholesterol in plasma lipoproteins were similar in labeled plasma samples. This method of labeling can be used for the measurement of cholesterol esterification, or lecithin:cholesterol acyltransferase activity, in small amounts (20–40 μl) of plasma samples. —**Yen, F. T., and T. Nishida.** Rapid labeling of lipoproteins in plasma with radioactive cholesterol. Application for measurement of plasma cholesterol esterification. *J. Lipid Res.* 1990. **31:** 349–353.

Supplementary key words lysophosphatidylcholine • micelles • lipoproteins • lecithin:cholesterol acyltransferase

Methods of labeling lipoproteins in plasma with radioactive cholesterol commonly utilize radioactive cholesterol dissolved in organic solvents (1, 2) or cholesterol associated with carriers (3–10), e.g., Celite. These methods are not effective when only small amounts of plasma are available. The amount of organic solvent, such as ethanol, used for the injection of radioactive cholesterol into plasma must be minimized in order to prevent the structural alteration of plasma lipoproteins. Also, when a carrier such as Celite is used (4, 5), it is difficult to obtain a uniform amount of Celite-lipid mixture for consistent labeling. Radioactive cholesterol in an albumin-stabilized emulsion (8–10) has been another commonly used method; however, an incubation time of 2–4 h (10) is required to equilibrate the radioactive cholesterol.

We developed a method for labeling plasma with radioactive cholesterol using lysophosphatidylcholine (lysoPC) micelles as the carrier. High affinity of lysoPC for cholesterol has been previously demonstrated (11). When injected into plasma, the lysoPC micelles would readily disintegrate as a result of the binding of lysoPC to plasma components, especially albumin (12, 13). This disintegration results in rapid equilibration of [^{3}H]cholesterol released from the micelles among lipoproteins in human plasma. The method was used for the determination of the activity of lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43), a cholesterol-esterifying enzyme in plasma (14–16).

EXPERIMENTAL PROCEDURE

Collection of plasma

Fresh blood samples were obtained from human volunteers after a 12-h fasting period by use of the finger puncture device, Autolet (Ulster Scientific Inc., Highland, NY). Blood (50–100 μl) was collected into heparinized microhematocrit capillary tubes (Baxter Healthcare Corp., McGaw Park, IL). Plasma was immediately separated, collected, and used in the experiments.

Preparation of [^{3}H]cholesterol-lysoPC micelles

Egg yolk lysoPC (1-acyl-*sn*-glycero-3-phosphocholine) of 99% purity was purchased from Sigma Chemical Co. LysoPC (0.2 μmol) in ethanol and [^{3}H]cholesterol (sp act, 5 Ci/mmol, NEN DuPont, Boston, MA) (4 nmol) in toluene were added into a 12 × 75 mm conical tube. Organic solvents were removed using a rotary evaporator. Complete removal of the organic solvents was necessary in order to obtain a stable micelle preparation. Since it was extremely time-consuming to remove trace amounts of toluene, we later replaced the toulene in the [^{3}H]cholesterol preparation with chloroform, a more volatile organic solvent. The toluene was removed with a rotary evaporator; to ensure complete removal, ether was added and evaporated off three times, followed by redissolving [^{3}H]cholesterol in chloroform. The sample containing [^{3}H]cholesterol and lysoPC was dried, 250 μl of 39 mM sodium phosphate buffer (ionic strength 0.1, pH 7.4) containing 0.025% EDTA was added, and the preparation was immediately vortexed. The micelle preparation was left standing at room temperature (20°C) for 2 h with intermittent vortexing. Recovery of radioactivity was >95%. When the [^{3}H]cholesterol-lysoPC micelle preparations were stored at 4°C overnight, some turbidity developed. This turbidity was not due to the presence of cholesterol; a similar degree of turbidity was observed in lysoPC micelles without cholesterol. Since the turbidity disappeared by bringing the preparations to room temperature, the micelles were usually kept at room temperature under nitrogen. We customarily used freshly prepared micelles for the experiments. A typical preparation contained 0.8 nmol of lysoPC and 16 pmol of [^{3}H]-cholesterol (0.08 μCi) per μl of micelle preparation (molar ratio, lysoPC:cholesterol, 50:1).

Abbreviations: lysoPC, lysophosphatidylcholine; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

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[^{3}H]Cholesterol labeling of plasma samples

To label plasma samples with [^{3}H]cholesterol, 5 μl of the micelle preparation was injected into 40 μl of fresh plasma containing the LCAT inhibitor, 1.4 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and incubated under N_2 at 37°C. Incubation times varied in each experiment. For the purpose of comparison, plasma samples were labeled in the same manner but with 12 μl of [^{3}H]cholesterol-albumin emulsion. The emulsion was prepared according to Stokke and Norum (10) by adding [^{3}H]cholesterol (4 μCi), dissolved in acetone, into 1 ml of 5% bovine serum albumin solution and by evaporating off acetone under a stream of N_2.

Dextran sulfate-$MgCl_2$-precipitation

The procedure for the precipitation of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) in plasma by the addition of dextran sulfate and $MgCl_2$ (17) was modified for smaller amounts of plasma. In a typical procedure, 5 μl of plasma samples were placed into small tubes (6 × 50 mm) kept in ice and were diluted with 86 μl of phosphate buffer. The VLDL + LDL fraction precipitated upon addition of 8 μl of 0.1% dextran sulfate (mol wt 500,000, Pharmacia P-L Biochemicals, Piscataway, NJ) and 1 μl of 2 M $MgCl_2$ (final concentration, 20 mM). After a 20-min standing period at 4°C, the supernatant and precipitate were separated by centrifugation (2,000 *g*, 15 min). The supernatant was removed and the precipitate was dissolved by the addition of 100 μl of 0.1 N NaOH. The radioactivities in the aliquots of the supernatant and precipitate fractions were determined in a Beckman LS250 scintillation spectrometer.

Separation of lipoprotein classes by gel permeation chromatography

Gel permeation chromatography was performed on a column of Superose 6B (1 × 32.5 cm) (Pharmacia P-L Biochemicals). The column was equilibrated with the elution buffer, 39 mM sodium phosphate buffer containing 0.025% EDTA, 0.06 M NaCl, and 2 mM NaN_3 (ionic strength, 0.16). Samples were applied to the column and were eluted at a flow rate of 18 ml/h which was maintained by the use of a peristaltic pump. Fractions of 0.6 ml were collected using a fraction collector and were analyzed for radioactivity to obtain the elution profile of the plasma lipoproteins. Peak areas of the major lipoprotein fractions were measured using a planimeter. Recovery of total radioactivity was >95%.

Separation of lipoprotein classes by density gradient ultracentrifugation

The method of Chung et al. (18) was modified for the smaller volume of samples used. Sucrose was used rather than potassium bromide to provide a more stable gradient. The samples were placed in Beckman Quick-Seal ultracentrifuge tubes, and were centrifuged in a Beckman vertical VTi80 rotor at 75,000 rpm (354,000 *g*) for 3.5 h at 4°C. Control tubes containing phosphate buffer instead of plasma were layered in a similar manner and centrifuged with each run. The slowest acceleration and deceleration run programs of the Beckman L8-80 ultracentrifuge were used. After centrifugation, a saturated sucrose solution was pumped at the rate of 10 ml/h into the bottom of the tube and 250-μl fractions were collected through tubing attached to the top of the centrifuge tube. Fractions (200 μl) in Biocount were analyzed for radioactivity to obtain the plasma lipoprotein profile.

Determination of LCAT activity

The activity of LCAT was measured in plasma samples labeled with [^{3}H]cholesterol. The plasma was preincubated at 37°C with the [^{3}H]cholesterol-lysoPC micelles or [^{3}H]cholesterol-albumin emulsion in the presence of 1.4 mM DTNB. After this preincubation period, 2-mercaptoethanol was added to a final concentration of 11.7 mM to reactivate plasma LCAT. The plasma was incubated at 37°C under N_2. Incubation periods varied among experiments. Control tubes contained only [^{3}H]cholesterol-labeled plasma and 1.4 mM DTNB throughout the experiment.

At predetermined time intervals, aliquots were removed and placed immediately in an ice bath. The amount of [^{3}H]cholesteryl esters was analyzed as previously described (19). Briefly, lipids were separated by organic solvent extraction, dried, and separated by thin-layer chromatography. The radioactivity in the [^{3}H]cholesterol and [^{3}H]cholesteryl ester spots was analyzed in a toluene scintillation mixture. The rate of cholesterol esterification is expressed as percent of free cholesterol esterified, or the percent of [^{3}H]cholesteryl esters relative to the total amount of radioactivity.

Enzymatic determination of free cholesterol

The method of Gamble et al. (20) was modified for the enzymatic determination of free cholesterol in small amounts of plasma. Although cholesterol oxidase preparations (Boehringer Mannheim Biochemicals) contained a trace of cholesterol esterase activity, it did not significantly influence the determination of free cholesterol. We noted that the cholesterol oxidase preparations from some companies contained substantial amounts of cholesterol esterase and were not suitable for free cholesterol determination. Cholesterol standards were obtained from Boehringer Mannheim Biochemicals.

RESULTS AND DISCUSSION

A method for labeling plasma lipoproteins with radioactive cholesterol was developed using [^{3}H]cholesterol-lysoPC micelles. The incorporation of radioactive cholesterol from [^{3}H]cholesterol-lysoPC micelles into plasma lipoproteins was conveniently traced by precipitating the VLDL and LDL fraction with dextran sulfate and $MgCl_2$ and determining the distribution of [^{3}H]cholesterol in the supernatant containing HDL and in the precipitate. The injection of [^{3}H]cholesterol-lysoPC micelles into plasma and subsequent incubation at 37°C in the presence of DTNB resulted in a progressive increase in the amount of radioactivity in the VLDL and LDL fraction, giving near maximum incorporation after a 20-min incubation period (**Table 1**). No appreciable change occurred during further incubation of the plasma samples. In contrast, when the [^{3}H]cholesterol-albumin emulsion was substituted for the [^{3}H]cholesterol-lysoPC micelles, the amount of [^{3}H]cholesterol in the VLDL and LDL fractions did not reach a plateau after 20 min of incubation, and continued to increase gradually upon further incubation for a total incubation period of 120 min.

It has been reported that lysoPC associates with albumin in a molar ratio of 1:1 (12) or 2:1 (13). Since plasma contains a high concentration of albumin, only a portion of the albumin present in the plasma would be required to bind the lysoPC component of the micelles. Thus, the addition of the [^{3}H]cholesterol-lysoPC micelles into plasma could lead to the disintegration of the micelles as a result of the binding, to albumin, of free lysoPC that is in equilibrium with micelle lysoPC. This would result in the rapid release of radioactive free cholesterol for incorporation into plasma lipoproteins. Our data suggest that [^{3}H]cholesterol from lysoPC micelles was incorporated into lipoproteins more efficiently than that from albumin emulsions, in which at least a portion of the cholesterol may exist in the form of aggregates and may equilibrate with lipoprotein cholesterol more slowly.

TABLE 1. Distribution of [^{3}H]cholesterol label in VLDL + LDL fraction[a]

	[^{3}H]Cholesterol in VLDL + LDL Fraction	
Incubation Time	LysoPC Micelles	Albumin Emulsion
min	%	
0	29.5 ± 3.25[b]	22.2 ± 1.05
10	51.7 ± 3.85	
20	70.8 ± 1.0	58.5 ± 3.25
40	73.2 ± 0.9	62.9 ± 0.7
60	72.7 ± 2.15	64.9 ± 1.1
120	68.0 ± 2.45	68.4 ± 0.7

[a][^{3}H]Cholesterol-lysoPC micelles or [^{3}H]cholesterol-albumin emulsions were injected into 40 μl of fresh human plasma samples containing 1.4 mM DTNB. The samples were incubated at 37°C for 2 h and aliquots equivalent to 5 μl of plasma were removed at various time intervals. The VLDL + LDL fraction was precipitated in the presence of dextran sulfate and $MgCl_2$. The supernatant containing HDL and the precipitate were analyzed for radioactivity.

[b]The percent of [^{3}H]cholesterol label in the precipitate containing the VLDL and LDL fractions is given as mean ± standard deviation from a minimum of four determinations.

To compare the distribution of radiolabeled free cholesterol and that of unlabeled free cholesterol in lipoprotein fractions of [^{3}H]cholesterol-labeled plasma, VLDL, LDL, and HDL were separated by gel permeation chromatography on a Superose 6B column. A control run was carried out using unlabeled plasma samples. The degree of separation of VLDL, LDL, and HDL was similar to that obtained by Chang, Hopkins, and Barter (21). The percentage of [^{3}H]cholesterol in the VLDL, LDL, and HDL fractions was comparable to the percentage of free cholesterol in these three fractions obtained from both labeled and unlabeled plasma samples (**Table 2, A**). Apparently, [^{3}H]cholesterol was equilibrated with free cholesterol in the lipoprotein fractions. Furthermore, the comparable distribution of free cholesterol in both labeled and unlabeled samples suggests that the procedure for labeling did not alter the distribution of native cholesterol among VLDL, LDL, and HDL.

Density gradient ultracentrifugation was also used to measure the distribution of [^{3}H]cholesterol among the three major lipoprotein classes in plasma labeled with [^{3}H]cholesterol. Typical profiles of the VLDL, LDL and HDL separation were similar to those obtained by Chung et al. (18). The relative distributions of [^{3}H]cholesterol among VLDL, LDL, and HDL separated by either gel permeation chromatography or density gradient ultracentrifugation were comparable (Table 2, B). It appears that the labeling of plasma by using [^{3}H]cholesterol-lysoPC micelles permits the determination of the [^{3}H]cholesterol equilibrium distribution, which essentially represents the free cholesterol distribution. This method would be useful in determining the free cholesterol of lipoproteins in limited amounts of plasma samples (20–40 μl), for which analysis is not possible by conventional methods.

The labeling method was applied to the determination of LCAT activity in plasma samples. Although lysoPC is known to inhibit LCAT activity, the amount of albumin in plasma is sufficiently high to overcome any inhibitory effect exerted by the lysoPC of the micelle preparation (13). To determine LCAT activity, plasma and [^{3}H]cholesterol-lysoPC micelles were preincubated in the presence of the LCAT inhibitor, DTNB, for varying periods. After preincubation, the LCAT in the plasma was reactivated by the addition of 2-mercaptoethanol, and its activity was measured after incubation periods of 15, 30, and 60 min (**Fig. 1**). A linear rate of cholesterol esterification was obtained for the samples that were

TABLE 2. Distribution of [^{3}H]cholesterol and unlabeled free cholesterol among lipoproteins[a]

Fractions	Cholesterol		
	VLDL	LDL	HDL
		%	
A. [^{3}H]Cholesterol-plasma			
[^{3}H]Cholesterol	7.0 ± 3.2[b]	62.8 ± 4.2	30.1 ± 5.7
Free cholesterol	10.0 ± 2.9	62.0 ± 2.8	28.0 ± 5.2
Unlabeled plasma free cholesterol	8.1 ± 1.1	60.2 ± 2.2	31.6 ± 2.0
B. Density gradient ultracentrifugation (n = 3)	7.4 ± 2.4	65.3 ± 2.0	25.2 ± 1.1
Gel permeation chromatography (n = 5)	5.4 ± 1.9	60.2 ± 3.7	34.1 ± 3.2

[a]In experiment A, plasma samples were incubated with the [^{3}H]cholesterol-lysoPC micelles at 37°C for 30 min in the presence of 1.4 mM DTNB. [^{3}H]Cholesterol-labeled plasma samples were applied to a Superose 6B column. Fractions eluted from the column were analyzed for [^{3}H]cholesterol and free cholesterol to determine their distribution in VLDL, LDL, and HDL. The analysis of free cholesterol in lipoprotein fractions separated from corresponding unlabeled plasma samples is also shown. In experiment B, plasma samples from a single donor were treated as in experiment A. Lipoprotein fractions were separated by gel permeation chromatography or density gradient ultracentrifugation. The percent of [^{3}H]cholesterol in VLDL, LDL, and HDL was obtained from the distribution of the radioactivity in the lipoprotein peaks.
[b]Values represent mean ± SD.

preincubated for 30, 60, and 120 min. The samples that were preincubated for 0 and 15 min showed higher apparent rates of esterification during the assay incubation period. These results suggest that a 30-min preincubation time is necesssary to obtain the equilibration of [^{3}H]cholesterol in plasma lipoproteins and hence a linear response of LCAT activity during the assay incubation. Unlike the present method, the albumin emulsion method for labeling requires a minimum preincubation period of 2 h and a recommended period of 4 h (10). Such a long preincubation period may result in alterations of the plasma lipoproteins by DTNB (7), HDL conversion factors (22, 23), proteolytic enzymes (24), and other unknown factors. We observed that plasma LCAT activity measured by the [^{3}H]cholesterol-albumin emulsion method gives a value which is about 20% less than that obtained by the present method. The short preincubation period, 30 min, required in our micelle injection method minimizes alterations of the plasma lipoproteins and permits the sensitive assay of plasma LCAT activity in very small amounts of plasma.

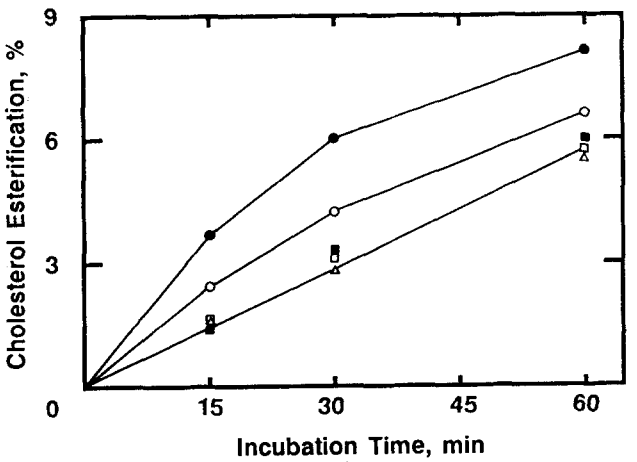

Fig. 1. Effect of preincubation time on LCAT activity in human plasma. Plasma samples containing [^{3}H]cholesterol-lysoPC micelles were preincubated for 0 (●), 15 (○), 30 (■), 60 (□), and 120 (△) min in the presence of 1.4 mM DTNB. LCAT activity was reactivated by the addition of 2-mercaptoethanol to a final concentration of 11.7 mM. The samples were incubated at 37°C and aliquots were removed at selected time intervals. [^{3}H]Cholesterol and [^{3}H]cholesteryl esters were extracted, separated by thin-layer chromatography, and analyzed for radioactivity. Enzyme activity is expressed as percent free cholesterol esterification.

The initial rate of cholesterol esterification can be determined without using radioactive cholesterol by measuring the decrease in free cholesterol mass in plasma samples incubated at 37°C. Patsch, Sailer, and Braunsteiner (25) described such a procedure using an enzymatic assay that requires 200 μl of plasma samples per assay. This amount is considerably greater than that used in the present method. Furthermore, the precise determinations of the initial rate, which amounts to only about a 3% decline in free cholesterol mass during a 30-min assay incubation period, cannot be easily attained by any nonradioactive method. To determine the initial rate with radioactive cholesterol, the label must be equilibrated with free cholesterol of plasma lipoproteins (26). Since this equilibration was obtained in our procedure, the percent free cholesterol esterification is likely to represent the decline in free cholesterol mass. It appears that the present method is useful for the determination of the initial rate of cholesterol esterification when only small amounts of plasma are available.

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