Influx, Efflux, and Hydrolysis of Cholesteryl Ester in Atheromatous Lesions of Cholesterol-Fed Rabbits

Jane L. Hough and Donald B. Zilversmit

Total plasma lipoproteins were labeled with radioactive cholesteryl ester or cholesteryl ether by transfer of these lipids from phosphatidylcholine vesicles in the presence of plasma lipid transfer activity. Intravenous injection of these preparations into hypercholesterolemic rabbits showed disappearance curves identical to those of in vivo labeled lipoproteins. Disappearance of cholesteryl ester and ether were similar during the first 24 hours, but they diverged at later time intervals, indicating recirculation of labeled cholesteryl ester. Lipoproteins labeled with cholesteryl ether were injected at 25 days, 7 days and 1 day before sacrifice of the rabbits. The maximal loss of labeled ether from the aortas during a 24-hour period ranged from 1.6% to 8.9% of the labeled ether taken up from plasma. Hydrolysis of cholesteryl ester by the artery during 24 hours averaged 35% of the calculated cholesteryl ester influx. After hydrolysis, cholesteryl ester fatty acid appeared to be esterified more rapidly than the cholesterol moiety of the cholesteryl ester.

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Accumulation of cholesteryl ester is the primary feature of the atherosclerotic lesion. Most of this cholesteryl ester is derived from plasma,1,2 and its accumulation is governed by the rates of arterial influx and efflux. The influx and metabolism of cholesteryl ester in normolipidemic and hypercholesterolemic rabbits has been studied with isotopically labeled cholesteryl esters.3–5 In such studies, quantification of the influx of plasma cholesteryl esters into tissues and its subsequent hydrolysis and re-esterification is difficult because once the cholesteryl ester is hydrolyzed, the resulting labeled unesterified cholesterol can readily exchange with unlabeled cholesterol outside the tissue. Recently, Stein et al.7 reported the use of cholesteryl ether as a nondegradable cholesteryl ester analogue to measure cholesteryl ester influx into arteries from normolipidemic and hypercholesterolemic rabbits. Since cholesteryl ether is stable and not hydrolyzed by enzymes that are present in tissues,8–12 it should be retained by cells in tissue and thus represent the total cholesteryl ester taken up. However, some studies13, 14 have reported that cholesteryl ether is slowly released unhydrolyzed by hepatocytes into the bile. Spleen and Kupffer cells, on the other hand, retain the cholesteryl ether for long periods of time. It is not known whether cholesteryl ether is retained by arterial tissue or whether it is subject to turnover, as has been reported for cholesterol in the atherosclerotic lesion of rabbit aorta.2,15

In the present study, we first evaluated the efflux of cholesteryl oleyl ether from artery, liver, and adrenal tissue and validated its use as representative of the total plasma cholesteryl ester arterial influx in vivo. Second, using isotopically labeled cholesteryl ether in conjunction with isotopically labeled cholesteryl oleate, we measured in vivo the tissue cholesteryl ester hydrolysis and esterification.

Methods

Animals

All rabbits were female New Zealand Whites from either Dutchland Laboratories, Denver, Pennsylvania, or Beckens Research Animal Farm, Sanborn, New York. Rabbits were fed daily 100 g of Purina

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Rabbit Laboratory Chow supplemented with 0.5 g cholesterol (Nutritional Biochemical Company, Cleveland, Ohio) and 2.5 g Wesson oil (Hunt Wesson Foods, Inc., Fullerton, California) or 0.2 g cholesterol and 2.0 g Wesson oil. Experimental protocols were approved by University guidelines.

**Radionuclide Compounds**

1,2(n)-3H-cholesterol (54 Ci/nmol) and 4-C-14-cholesterol (58.4 mCi/nmol) were purchased from Amer sham Corporation (Arlington Heights, Illinois); cholesteryl 1-14C-oleate from New England Nuclear (Boston, Massachusetts). We synthesized 3H-cholesteryl oleate from 3H-cholesterol and oleoyl chloride (Nu-Chek Prep Incorporated, Elysian Minnesota) as described by Goodman.18 The reaction mixture was chromatographed on a Woelm (ICN, Cleveland, Ohio) neutral aluminum oxide column (1 g aluminum oxide (Woelm Supra I, deactivated to 5% H2O) to 10 mg lipid). The column was washed with 10 to 20 ml of hexane (Skelly Solve B (redistilled), Getty Refining and Marketing Company, Tulsa, Oklahoma), 1% diethyl ether in hexane, and 3% diethyl ether in hexane. Cholesteryl ester was then eluted with 20 ml of 5% diethyl ether in hexane. 3H- and 14C-cholesteryl oleyl ether were synthesized from 3H- and 14C-cholesterol and oleoyl methane sulfonate.17 Radiolabeled cholesterol was added to a small vial, and the solvent was removed under a stream of nitrogen. An excess of oleyl methane sulfonate in dry benzene and 3 to 4 drops of a suspension of potassium hydroxide in mineral oil were added. The vial was flushed with nitrogen, closed, and maintained at about 60° C overnight. Reaction products were chromatographed on a Woelm neutral aluminum oxide column. The column was washed with 10 to 20 ml of hexane and 9:1 hexane/cyclohexane. Cholesteryl ether then was eluted with 8:2 hexane/cyclohexane.

The purity of each isotopic compound was determined by thin-layer chromatography (TLC) on precoated silica gel plates (Silica Gel 60, E. Merck, Darmstadt, Germany) with hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol) as the developing solvent. Compounds with a radiopurity of less than 98% were purified by TLC in hexane/diethyl ether (70:30, vol/vol) and eluted with chloroform/methanol (9:1, vol/vol).

**Experimental Procedure**

Plasma was labeled by incubation with egg phosphatidycholine (Lipid Products, South Nutfield, England) vesicles containing radioactive cholesteryl oleyl ether and cholesteryl oleyl ether as described previously.18,19 Labeled cholesteryl oleate or labeled cholesteryl oleyl ether and egg phosphatidycholine (1:5 mol/mol) were added in chloroform to a 50 ml glass tube, dried under a stream of N2 at 37°C, and any residual solvent was removed under reduced pressure on a rotary evaporator for 10 minutes. Then 2 ml of 50 mM phosphate buffer (pH 7.4) was added to the dried lipids. The tube was flushed with N2 and mixed on a vortex mixer for 10 minutes to resuspend the lipids. The resulting suspension was sonicated at full power for 40 minutes in a bath type sonicator (Laboratory Supplies Company, Hicksville, New York). Phosphatidycholine vesicles were added to plasma collected from animals after a 24-hour fast, so that the added phosphatidycholine did not exceed 7% of the total phospholipid content. Also present were diethyl p-nitrophenyl phosphate (1 mM) and 0.01 ml of 0.4 M disodium EDTA, 4% NaN3 per milliliter of liposome-sample mixture. This mixture was incubated at 37°C for 18 to 24 hours at which time no intact vesicles remained as judged by initial disappearance rates from the circulation after intravenous dosage. Before injection, labeled plasma was dialyzed against 0.15 m NaCl at 4°C for 24 to 48 hours and passed through a 0.22 µm filter (Milllex GS, Millipore Corporation, Bedford, Massachusetts).

At the end of the cholesterol-feeding period, labeled autologous plasma was injected into the marginal ear vein of treated and untreated rabbits. Serial blood samples were taken from the other ear vein with 0.4 M EDTA (0.01 ml/ml blood) as an anticoagulant. Some rabbits were also injected intravenously with autologous, 3H-cholesteryl oleyl ether-labeled plasma 7 days or 25 days before the end of the experiment. The animals were anesthetized with 25 mg nembutal/kg intravenously, and the vascular bed was perfused with at least 0.5 liter of cold 0.15 M NaCl. The saline entered the left ventricle and emerged from the severed inferior vena cava. Tissues were rinsed with cold saline, weighed, and frozen until analyzed. The aorta from the arch to just below the diaphragm was removed and dissected free of adventitia. The intima and inner media layers were stripped from the remaining media and frozen until analyzed. Plasma was centrifuged in a Beckman 40.3 rotor at 4°C, for 1.58 x 108 g x minutes. Very low density lipoproteins (VLDL) were isolated at d < 1.006, intermediate density lipoproteins (IDL) at 1.006 < d < 1.019, low density lipoproteins (LDL) at 1.019 < d < 1.063, and high density lipoproteins (HDL) at d > 1.063.

**Lipid Analysis**

All plasma and lipoprotein samples were extracted by partitioning into hexane from 42% ethanol in water according to the method of Thompson et al.20 Aliquots of the hexane phase were used for determination of total radioactivity and for separation into nonesterified and esterified cholesterol by thin-layer chromatography on precoated plates (Silicagel 60, E. Merck, Darmstadt, Germany) with hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol) as the developing solvent. Under these conditions, the Rf of cholesteryl ester and cholesteryl ether were similar. Nonesterified and esterified cholesterol fractions were eluted from the silica gel with chloroform/methanol (9:1, vol/vol). Aliquots were taken for determination of radioactivity and chole-
terol mass after saponification. Radioactivity was determined in a toluene scintillator containing 2% ethanol. When no analysis of cholesterol mass was needed, the silica gel was scraped directly into vials with ACS scintillator containing 2% ethanol (Amersham Corporation). Since cholesteryl ether is not saponified under the conditions described by Abell et al., separation of cholesteryl ester and cholesteryl ether containing the same radiolabel was accomplished by saponification of the cholesteryl ester fraction from TLC, with subsequent separation of the labeled cholesterol and cholesteryl ether on TLC.

Tissue samples were minced in a small amount of methanol and extracted for 24 hours at room temperature with at least 20 volumes of chloroform/methanol (1:1, vol/vol). The protein was pelleted by centrifugation, the solvent was removed, and the protein pellet was washed three times with 3 ml portions of chloroform/methanol (1:1, vol/vol). The washes were added to the original extract, and the solvent composition was adjusted to 2:1 (vol/vol) chloroform/methanol. Water was added to make up 20% of the final volume, and the top phase was removed and discarded. Aliquots of the chloroform phase were used for determination of total mass and radioactivity and for separation of unesterified/esterified cholesteryl and cholesteryl ether, as described for plasma and lipoprotein samples.

**Calculations**

The total influx of esterified cholesterol was calculated by dividing the tissue cholesteryl ether radioactivity by the area under the plasma cholesteryl ether "specific activity" curve, from the time of injection to the termination of the experiment. The "specific activity" was the ratio of the cholesteryl ether label to the esterified cholesterol mass. The units of this influx (mass/unit time) have usually been divided by the concentration of cholesteryl ester in plasma to give influx as a plasma clearance, i.e., ml of plasma/unit of time.

The hydrolysis of newly entered esterified cholesterol was determined from the difference between total influx, as measured from cholesteryl ether, and the net influx of cholesteryl ester, as measured from the tissue esterified cholesterol radioactivity, divided by the area under the plasma esterified cholesterol specific activity curve. The latter represents the amount of cholesteryl ester taken up that was not hydrolyzed during the experimental period. To determine the fraction of cholesteryl ether lost from a tissue, we injected H-cholesteryl ether at t, and C-cholesteryl ether at t₂. We calculated the influx (ml/h) of C-cholesteryl ether as cpm C14 in tissue divided by the area under the plasma C14 concentration curve during 24 hours after C14 injection. From this, we calculated the expected tissue cpm of H-cholesteryl ether, if no loss had occurred, by multiplying influx with the area under the H14 concentration curve. The difference between the expected and observed tissue H-cholesteryl ether can be ascribed to loss of tissue H-cholesteryl ether during the experimental period. Alternatively, this difference could be due to growth of the atheromatous lesion and a resulting increase of plasma lipoprotein influx into the tissue at t₂ (see Reference 2 for such an increased influx in the case of cholesteryl ester). If increased influx should occur, the loss of cholesteryl ether from the tissue may be even smaller than that calculated here.

**Results**

In our present study, the isotopically labeled cholesteryl olate and cholesteryl oleyl ether were incorporated into lipoproteins by incubating them at 37° C for 12 to 24 hours with phosphatidylcholine vesicles containing 16 mol% cholesteryl ester. Incorporation of labeled cholesteryl ester into lipoproteins by this procedure was representative of in vivo incorporated labeled cholesteryl esters, as shown by parallel plasma clearance in normal rabbits. We also found this to be true in the present study when lipoproteins were injected into hypercholesterolemic rabbits.

Figure 1 shows the typical slow clearance, early after injection, of labeled cholesteryl esters of plasma labeled in vivo and then in vitro by incubation with phosphatidylcholine vesicles. The disappearance rates of the two cholesteryl ester labels, even immediately after injection, were identical. If a significant quantity of intact vesicles had been present in the injected dose, these would have disappeared much more rapidly than the labeled lipoproteins. The distribution of the cholesteryl ester and ether labels into d < 1.019 and d > 1.019 lipoproteins of recipient animals was the same. Figure 2 shows that, during a 24-hour period, cholesteryl ether disappears from plasma, as does cholesteryl ester, and is, therefore, representative of plasma cholesteryl ester during this time. However, the apparent divergence of the plasma disappearance curves at 24 hours, which was not significant at the 5% level by t test, steadily increased over an 11-day period (Figure 3). The

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Clearance of cholesteryl ester-labeled hypercholesterolemic plasma (4.5 ml) injected intravenously into hypercholesterolemic rabbits. Autologous plasma was labeled in vivo (Δ) and then in vitro (○) by incubation with phosphatidylcholine vesicles at 37° C for 18 hours.
plasma disappearance curve for cholesteryl ester leveled off during the experimental period, whereas the plasma cholesteryl ether concentrations continued to decrease. This suggests that labeled cholesteryl ester was more rapidly resecreted into plasma than cholesteryl ether.

For estimation of the efflux of cholesteryl ether from various tissues, we injected rabbits with autologous plasma labeled with $^3$H-cholesteryl oleyl ether.

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![Figure 2. Clearance of hypercholesterolemic plasma labeled with cholesteryl oleate (x) and cholesteryl oleyl ether (o). Plasma (5.5 ml) was injected intravenously into hypercholesterolemic rabbits.](image2)

![Figure 3. Extended clearance of cholesteryl oleate- (x, o) and cholesteryl oleyl ether- (Δ, □) labeled hypercholesterolemic plasma injected intravenously into two hypercholesterolemic rabbits.](image3)

Table 1. Cholesterol Content and Cholesteryl Oleyl Ether Influx into the Artery, Liver, and Adrenal of Hypercholesterolemic Rabbits

<table>
<thead>
<tr>
<th>Organ</th>
<th>Time between injections (hr)</th>
<th>Total cholesterol</th>
<th>Influx cholesteryl ether</th>
<th>Cholesteryl ether lost from tissue* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5% cholesterol diet for 18 weeks (3)</td>
<td>165</td>
<td>985 ± 199</td>
<td>28 ± 5</td>
<td>1.1 ± 0.2 2.6 ± 0.8 52 ± 5</td>
</tr>
<tr>
<td>0.2% cholesterol diet for 33 weeks (4)</td>
<td>602</td>
<td>691 ± 106</td>
<td>30 ± 4</td>
<td>1.2 ± 0.2 2.3 ± 0.2 46 ± 5</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5% cholesterol diet for 18 weeks (3)</td>
<td>165</td>
<td>985 ± 199</td>
<td>46 ± 3</td>
<td>12 ± 2 34 ± 5 64 ± 1</td>
</tr>
<tr>
<td>0.2% cholesterol diet for 33 weeks (4)</td>
<td>602</td>
<td>691 ± 106</td>
<td>24 ± 2</td>
<td>17 ± 3 41 ± 5 59 ± 2</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2% cholesterol diet for 33 weeks (4)</td>
<td>602</td>
<td>691 ± 106</td>
<td>164 ± 14</td>
<td>10 ± 1 19 ± 1 41 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± standard error. Number of animals is given in parentheses.

*The percent of cholesteryl ether lost from the artery was calculated from the difference between the predicted and observed cholesteryl ether content (see Calculations). This calculation assumes that during the interval between injections the influx remains constant.
Table 2. Cholesterol Content and Cholesteryl Ester and Ether Influx Into Artery and Liver of Hypercholesterolemic Rabbits

<table>
<thead>
<tr>
<th>Organ</th>
<th>Experimental period (hr)</th>
<th>Total cholesterol (mg/g tissue)</th>
<th>Influx of cholesteryl (µl/g/hr)</th>
<th>Cholesteryl ester hydrolyzed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artery</td>
<td></td>
<td></td>
<td>Ether Ester</td>
<td></td>
</tr>
<tr>
<td>0.5% cholesterol diet for 8 weeks (1)</td>
<td>7</td>
<td>8.8</td>
<td>0.62 0.5</td>
<td>19</td>
</tr>
<tr>
<td>0.5% cholesterol diet for 8 weeks (4)</td>
<td>24</td>
<td>20 ±8</td>
<td>1.7 ±0.6 1.1 ±0.4</td>
<td>39 ±4</td>
</tr>
<tr>
<td>0.2% cholesterol diet for 33 weeks (4)</td>
<td>24</td>
<td>30 ±4</td>
<td>2.3 ±0.2 1.5 ±0.1</td>
<td>32 ±3</td>
</tr>
<tr>
<td>0.5% cholesterol diet (2)</td>
<td>264</td>
<td>36 ±16</td>
<td>1.6 ±0.3 1.3 ±0.2</td>
<td>19 ±7</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5% cholesterol diet for 8 weeks (2)</td>
<td>4–7</td>
<td>81 ±13</td>
<td>47 ±20 32 ±16</td>
<td>35 ±6</td>
</tr>
<tr>
<td>0.5% cholesterol diet for 8 weeks (4)</td>
<td>24</td>
<td>64 ±8</td>
<td>18 ±3 9 ±2</td>
<td>50 ±8</td>
</tr>
<tr>
<td>0.2% cholesterol diet for 33 weeks (4)</td>
<td>24</td>
<td>24 ±2</td>
<td>41 ±5 16 ±2</td>
<td>61 ±2</td>
</tr>
<tr>
<td>0.5% cholesterol diet (2)</td>
<td>264</td>
<td>39 ±22</td>
<td>20 ±4 4 ±1</td>
<td>76 ±11</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the number of rabbits. Values are means ± standard error.

To assess the metabolic fate of cholesteryl ester after it entered the artery and liver, we injected the rabbits with autologous plasma that had been labeled with 3H-cholesteryl oleate, 14C-cholesteryl oleyl ether, or, in a few cases, with cholesteryl 14C-oleate. This indicates that once the cholesteryl ester in the artery did not change consistently as the influx period was increased from 7 to 264 hours. In contrast, the percent cholesteryl ester hydrolyzed by the liver steadily increased from a 7-hour to a 264-hour influx period. During a 24-hour period, the apparent hydrolysis of cholesteryl ester was one-third the hydrolysis of 3H-cholesteryl oleate. This indicates that once the cholesteryl oleate was hydrolyzed, the labeled oleic acid was more rapidly (66% in 24 hours) reused to esterify cholesterol in arterial tissue than was the cholesteryl component.

Discussion

Our present study confirms earlier reports10–15 on the stability of the ether bond and, as shown previously for the rat, establishes that in the cholesterol-fed rabbit, cholesteryl ether is representative of plasma cholesteryl ester.10–12 The lipoprotein distribution and plasma clearance of labeled cholesteryl ester and ether were not significantly different over a 24-hour period after intravenous injection. The significant divergence of the plasma disappearance curves occurring after 24 hours indicated that cholesteryl ether was not as readily resecreted by tissues such as liver as was cholesteryl ester. For cholesteryl ether to be representative of the total arterial influx, it has to be retained by the tissue during the experimental period. The loss of cholesteryl ether from arterial tissue was determined by injecting labeled cholesteryl ether at two different times and comparing the accumulation of the two labels. These values represent the maximum arterial efflux because the calculation assumes that the rate of influx remains constant during the interval between injections. If an increase in arterial influx had occurred, the efflux of cholesteryl ether would have been underestimated (see Methods). It has been shown that influx of cholesteryl ester into the artery increases with the growth of the lesions.2,7 which is exponential in the cholesterol-fed rabbit. It is probable that efflux of the ether over a 24-hour period is insignificant because the measured efflux of cholesteryl ether from the artery was only 1.6% to 8.9% for a 24-hour period, which probably overestimates the actual efflux. Influx and efflux of cholesteryl ether were measured in two groups of rabbits, fed 0.2% or 0.5% cholesterol diets for 33 or 18 weeks with time intervals between injections of 165 hours or 602 hours, respectively.

At termination of the experiments, the arterial cholesterol content was similar for the two groups. This indicates that the rabbits on the short-term, higher cholesterol diet (Group 1) developed arterial lesions at a faster rate than the rabbits on the long-term, lower cholesterol diet (Group 2). However, the total percent efflux of cholesteryl ether over 165 hours (Group 1) and 602 hours (Group 2) was similar for both groups of rabbits. This time-independence of percent "loss" was unexpected. In part, this might be
accounted for by the assumption that influx rates are constant over time. However, it is interesting that a similar independence of time is shown by the percent hydrolysis of cholesteryl ester.

The use of cholesteryl ether to measure the total plasma cholesteryl ester influx has enabled us to measure cholesteryl ester hydrolysis and esterification in vivo without complex design, as was used previously. The hydrolysis of cholesteryl ester was determined by comparison of the influx of labeled cholesteryl ether with the influx of labeled cholesteryl ester. Despite different experimental periods, only 19% to 39% of the newly entered cholesteryl ester was hydrolyzed in the artery. These results suggest that perhaps a constant portion of the cholesteryl ester taken up by the artery reaches the degradative site and is hydrolyzed. If only a constant portion is hydrolyzed, then the percent hydrolysis would remain constant over varying influx periods. Hydrolysis of cholesteryl ester by the liver increased as the experimental period increased, 35% for a 4- to 7-hour period to 76% for a 264-hour period, suggesting that all of the cholesteryl ester taken up by the liver reaches the site of degradation.

It is difficult to determine whether a net loss of hydrolyzed cholesteryl ester occurred, since unesterified labeled cholesterol in tissue may exchange readily with extracellular and plasma unesterified cholesterol. There was no loss of labeled unesterified cholesterol from the artery, but 0 to 90% was lost from the liver, increasing as the experimental period increased. The influx of cholesteryl ester into arterial tissue reported here (0.38–0.99 µg/cm²/hr) is comparable to that reported by Stein et al. (0.46–2.39 µg/cm²/hr for intima and media) and by Stender and Zilversmit (0.45–2.15 µg/cm²/hr). The fact that the duration of these influx studies differed, from 6 hours with cholesteryl ester (Stender and Zilversmit) to 14 days (Stein et al.) with cholesteryl ether, supports our suggestion that efflux of cholesteryl ether from the artery is insignificant. The percent hydrolysis reported here was also similar to that reported by Zilversmit and Stein. However, the percent esterification was higher in the present study. This difference is probably due to the method used to measure esterification. Stender and Zilversmit used radiolabeled cholesterol taken up by the artery from the plasma.

The present study compared the hydrolysis of 3H-cholesteryl ester and cholesteryl 14C-oleate to measure the esterification of cholesterol within tissues. Studies have shown that enhanced sterol esterification occurs during atherogenesis and have suggested that esterification leads to cellular deposition. It has also been shown that in vitro free fatty acids penetrate to the esterifying sites of the aorta more readily than cholesterol. We have found that in vivo oleic acid is more readily reused for cholesterol esterification than the corresponding cholesterol. This may be due to the ability of the oleic acid to reach the esterifying site faster, or to a larger pool of unlabeled unesterified cholesterol present in the artery which may be available for esterification. Therefore, esterification may be underestimated when radiolabeled cholesterol is used.

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References


Index Terms: cholesteryl ester influx in atheroma • cholesteryl ester hydrolysis in atheroma • cholesteryl ether influx in atheroma • plasma lipoprotein labeling • plasma lipoprotein clearance
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