

Partial characterization of low density lipoprotein preparations isolated from fresh and frozen plasma after radiolabeling by seven different methods

Douwe E. Atsma,^{1,*} Herman Jan M. Kempen,^{*} Willem Nieuwenhuizen,^{*} Ferdinand M. van 't Hooft,[†] and Ernest K. J. Pauwels^{**}

Gaubius Institute TNO,^{*} P.O. Box 612, 2300 AP Leiden; Department of Cardiology[†] and Department of Nuclear Medicine,^{**} University Hospital Leiden, The Netherlands

Abstract Four ^{99m}Tc and three ¹²⁵I labeling methods were evaluated for their suitability to label low density lipoproteins (LDL) for the purpose of scintigraphic biodistribution studies. For ^{99m}Tc these methods were: direct incorporation in LDL of ^{99m}TcO₄⁻ using sodium dithionite (dithionite method); a method using first N,N-dimethylformamide to prepare a ^{99m}Tc-complex reacting with LDL in a subsequent step (DMF method); a technique in which ^{99m}TcO₄⁻ is first coupled to a diamide dithiolate derivative of pentanoic acid by reduction with dithionite, followed by coupling of this ligand to LDL (N₂S₂ method); and a method using sodium borohydride and stannous chloride as reducing agents (borohydride method). The iodination techniques were based on oxidation of I⁻→I⁺, using iodine monochloride (ICl method), 1,3,4,6-tetrachloro-3,6-diphenylglycoluril (Iodogen method), and N-bromosuccinimide (NBS method) as oxidants. We studied labeling yields, modification of LDL caused by the labeling procedures using agarose-gel electrophoresis, and radiochemical stability of the labeled LDL complex upon incubation in plasma at 37°C for 15 h. We used Sepharose CL6B chromatography to separate LDL from other plasma proteins. We also examined whether LDL isolated from frozen plasma (Pool-LDL) gave results similar to LDL obtained from freshly prepared plasma (Fresh-LDL). Pool-LDL radiolabeled by the dithionite, DMF, NBS, and Iodogen methods lost its label upon incubation with plasma. This also happened with Fresh-LDL when the DMF, NBS and Iodogen methods were used. Upon agarose-gel electrophoresis, no modification of LDL was observed with all methods when the radionuclide/LDL ratio was kept low. However, when higher ratios were used, the LDL was detectably modified by the DMF and Iodogen methods as evidenced by its increased electronegativity. For both Pool-LDL and Fresh-LDL, ^{99m}Tc-labeled LDL prepared by the N₂S₂ and borohydride method, and ¹²⁵I-labeled LDL as obtained by the ICl method were both stable and apparently unmodified. For the dithionite method this was also true when Fresh-LDL was used. The plasma clearance studies of these stable radiolabeled LDL preparations in rabbits showed similar clearance rates for ^{99m}Tc-labeled LDL as obtained by the N₂S₂ method and ¹²⁵I-labeled LDL as iodinated by the ICl method, for both Fresh- and Pool-LDL. ^{99m}Tc-labeled Fresh-LDL produced by the dithionite method showed a slightly accelerated plasma decay, whereas both Fresh- and Pool-LDL labeled by the borohydride method were cleared significantly faster from the circulation. **■** The better labeling results of Fresh-LDL as compared with Pool-LDL, and possible modification of Pool-LDL induced

during storage, suggests that the use of Fresh-LDL is preferable. The above data indicate that only Fresh-LDL labeled by the dithionite method, the N₂S₂ method, and the ICl method are potentially acceptable as scintigraphic agents in biodistribution studies.—Atsma, D. E., H. J. M. Kempen, W. Nieuwenhuizen, F. M. Van 't Hooft, and E. J. K. Pauwels. Partial characterization of low density lipoprotein preparations isolated from fresh and frozen plasma after radiolabeling by seven different methods. *J. Lipid Res.* 1991. 32: 173-181.

Supplementary key words Technetium • iodination • lipoprotein turnover • scintigraphy • biodistribution • labeling procedures

Scintigraphy after intravenous administration of gamma-emitting radiopharmaceuticals allows the study in vivo, in a relatively noninvasive way, of the behavior of the labeled substances in the circulation and tissues. This technique has recently been used to study the metabolism and tissue distribution of radiolabeled low density lipoprotein (LDL) (1-4) and to visualize the occurrence and location of atherosclerotic plaques using radiolabeled LDL as a scintigraphic agent (4-7). The most commonly used radionuclides in scintigraphy are ^{99m}Tc and ¹²⁵I, since these radionuclides combine a high emitted energy with a short half-life, resulting in high image quality and a low radiation dose to the patient.

Radiolabeling methods for LDL to be used in biodistribution studies should preferably have the following characteristics: *i*) specific activity of radiolabeled LDL should be sufficiently high to yield good quality scintigraphic images within a suitable time period; *ii*) the stability of the radiolabeled LDL complex, i.e., the attachment of the radionuclide to the LDL particle in circula-

Abbreviations: LDL, low density lipoproteins; DMF, N,N-dimethylformamide; N₂S₂, 4,5-bis(thioacetamido)pentanoate; TCA, trichloroacetic acid; NBS, N-bromosuccinimide; RES, reticulo-endothelial system; Pool-LDL, LDL isolated from a pool of frozen plasma; Fresh-LDL, LDL isolated from fresh plasma; HBS, HEPES-buffered saline.

¹To whom correspondence should be addressed.

tion, should be strong enough to allow a meaningful interpretation of the observed distribution of radioactivity; and *iii*) LDL should not be grossly modified as a result of the labeling method to ensure a behavior of labeled LDL comparable to that of unlabeled LDL. For the radiolabeled LDL particle that is to be used for localizing a specific site, e.g., an atherosclerotic plaque, the most important requirement is that it accumulates at the site to be studied.

It was the aim of this study to evaluate seven LDL labeling techniques with respect to the characteristics mentioned above for LDL to be used in biodistribution studies: four methods using ^{99m}Tc and three using ^{123}I . We also studied whether the use of LDL isolated from a pool of frozen plasma (being a convenient supply for the lipoprotein) gave results similar to those achieved using LDL isolated from freshly prepared plasma. Of the ^{99m}Tc labeling techniques we examined the method described by Lees et al. (6), who first reported on the radiolabeling of LDL, using sodium dithionite as a reducing agent. In addition, we examined the method using N,N-dimethylformamide described by Feitsma et al. (8), the diamide dithiolate chelating technique recently described by Fritzberg et al. (9), and a newly developed method using sodium borohydride (patent application filed). The latter three procedures have not previously been used for the radiolabeling of LDL. The three iodination techniques that we evaluated were: the iodine monochloride procedure described by McFarlane (10) and modified by Bilheimer, Eisenberg, and Levy (11), the most commonly used method for iodination of lipoproteins; the Iodogen® iodination method described by Fraker and Speck (12); and the method using N-bromosuccinimide as oxidizing agent, described by Sinn et al. (13) which is a simple and apparently highly efficient method for radioiodination of various proteins.

It was found that only Fresh-LDL, ^{99m}Tc -labeled by the dithionite method and the diamide dithiolate ligand method or iodinated by the iodine monochloride method, gave satisfactory results.

MATERIALS

N,N-dimethylformamide, trichloroacetic acid, sodium dithionite, glycine and acetonitrile were from Merck (Darmstadt, West Germany). N-Bromosuccinimide was from Sigma (St. Louis, MO). 4,5-Bis(benzoylthioacetamido)pentanoic acid was a generous gift from Dr. A. R. Fritzberg, NeoRx Corporation, Seattle, WA. 2,3,5,6-Tetrafluorophenol and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide were obtained from Aldrich (Milwaukee, WI). Potassium iodide was supplied by Nutritional Biochemical Corp. (Cleveland, OH). Sephadex G25 1 × 10 cm columns (PD 10) were purchased from Pharmacia (Uppsala, Sweden) and C_{18} cartridges were from J. T.

Baker Chemical Co. (Phillipsburg, NJ). $^{99m}\text{TcO}_4^-$ was obtained by elution with saline from a commercial $^{99}\text{Mo}/^{99m}\text{Tc}$ radionuclide generator, supplied by Mallinkrodt (Petten, Holland). Na^{123}I was obtained as a solution in 3–7 μl 0.05 M NaOH (sp act 5×10^5 mCi/mmol, carrier free) from Cigne (Eindhoven, Holland). 1,3,4,6-Tetrachloro-3 α ,6 α -diphenylglycoluril (Iodogen®) was obtained from Pierce Chemical Co. (Rockford, IL).

METHODS

LDL preparation

Human citrated plasma was obtained from the local blood bank. It was pooled, divided in portions, and stored at -80°C . For individual experiments LDL was isolated from a portion of this plasma (Pool-LDL). Alternatively, LDL was obtained from freshly prepared EDTA-plasma, obtained from normal healthy volunteers (Fresh-LDL). The LDL was isolated from the plasma by density gradient ultracentrifugation at 284,000 g for 18 h, as described by Terpstra, Woodward, and Sanchez-Muniz (14). The protein concentration of the LDL was quantitated by the method of Lowry et al. (15). LDL was harvested and used without further treatment.

Labeling procedures using ^{99m}Tc

^{99m}Tc labeling using sodium dithionite (dithionite method). The modification of this procedure described by Valabhajosula et al. (16) was used. LDL (protein concentration 1.3–2.4 mg/ml) and $^{99m}\text{TcO}_4^-$ were mixed in a ratio of 5–6 mCi/mg protein and 0.1 ml of a freshly prepared sodium dithionite solution (100 mg/ml glycine buffer, pH 10) was added. After incubation at room temperature for 30 min, complete separation of the ^{99m}Tc -labeled LDL from unbound $^{99m}\text{TcO}_4^-$ was achieved by means of size exclusion chromatography on a 10 × 1 cm Sephadex G25 column, prewashed with 4 mg albumin in 2 mg HBS buffer pH 7.4, using 20 mM HEPES-buffered saline (HBS), pH 7.4, as eluant. All Sephadex G25 columns used in our study were pretreated this way.

^{99m}Tc labeling using N,N-dimethylformamide (DMF method) (8). In this procedure, 200 μl of a $^{99m}\text{TcO}_4^-$ solution (20–80 mCi/ml saline) was added to a mixture of 11 μl of N,N-dimethylformamide and 3 μl 5 N hydrochloric acid and heated to 140°C for 4 h. After cooling, 200 μl chloroform was added. The clear contents of the tube were transferred to another tube and evaporated to dryness under a stream of warm air. Subsequently, 0.75–1.00 ml of LDL (protein concentration 1.46–3.12 mg/ml) was added and incubated for 1 h at 40°C . The radiolabeled LDL was separated from free ^{99m}Tc on a 10 × 1 cm Sephadex G25 column.

^{99m}Tc labeling using a diamide dithiolate ligand (N_2S_2 method). The method of Fritzberg et al. (9) was adapted as

follows. To a mixture of 5 μ l of 4,5-bis(benzoylthioacetamido)pentanoic acid [1.0 mg/ml solution in 90% (by vol.) CH_3CN] and 20 μ l of 1 M NaOH, 18–24 mCi $^{99\text{m}}\text{TcO}_4^-$ in 0.2–1 ml normal saline was added. After addition of 0.2 mg sodium dithionite (200 μ l of a freshly prepared 1 mg/ml solution in saline), the mixture was incubated for 15 min at 75°C. After lowering the pH by addition of 60 μ l of 0.2 M sodium phosphate buffer, pH 6.0, and 20 μ l of 1 M HCl, 20 μ l of 2,3,5,6-tetrafluorophenol [100 mg/ml solution in 90% (by vol.) CH_3CN] and 20 μ l of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide [125 mg/ml solution in 90% (by vol.) CH_3CN] were added. After incubation for 30 min at 75°C, the mixture was applied on a conditioned C_{18} cartridge. The cartridge was washed with 20 ml ethanol/0.01 M sodium phosphate buffer, pH 7, 2:8 (by vol.) and the tetrafluorophenyl ester of $^{99\text{m}}\text{Tc}$ -4,5-bis(thioacetamido)pentanoate was eluted with 100% CH_3CN . After evaporation of the CH_3CN under N_2 , 0.5 ml LDL (protein concentration 2.4–5.2 mg/ml) and 0.5 ml of 0.2 M sodium phosphate buffer, pH 9, were added. The mixture was incubated for 30 min at room temperature and applied to a 10 \times 1 cm Sephadex G25 column for separation of $^{99\text{m}}\text{Tc}$ -labeled LDL from unbound active ester.

$^{99\text{m}}\text{Tc}$ labeling using sodium borohydride (borohydride method) (patent application filed). This newly developed method will be described in further detail elsewhere. In short, to a mixture of $^{99\text{m}}\text{TcO}_4^-$, stannous chloride and sodium borohydride, LDL was added and incubated at room temperature for 2 h. Sodium citrate was then added and the mixture was allowed to stand for 0.5 h at room temperature. Separation of the $^{99\text{m}}\text{Tc}$ -labeled LDL from free $^{99\text{m}}\text{TcO}_4^-$ was performed using a 10 \times 1 cm Sephadex G25 column.

Labeling procedures using ^{123}I

Iodination using iodine monochloride (ICl method) (11). In this method, 1 ml of LDL (1.3–4.6 mg protein/ml) and 0.2 ml 1 M glycine in 0.25 M NaOH (pH 10) were added to a mixture of 10 mM ICl solution (7 μ l/mg protein), Na^{123}I (2.5 μ l/mg protein) and 1 M glycine in 0.25 M NaOH (25 μ l/mg protein). After incubation for 10 min at room temperature, ^{123}I -labeled LDL was separated from free ^{123}I on a 10 \times 1 cm Sephadex G25 column.

Iodination using 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril (Iodogen[®] method) (12). In this method, Iodogen[®] (25 μ l of a 2 mg/ml chloroform solution) was coated to a test tube by evaporation of the chloroform. To start the labeling, 1 ml LDL (2.41–2.89 mg protein/ml), 400 μ l HBS buffer (pH 7.4) and 20 μ l Na^{123}I (25 μ g/ml in 0.05 N NaOH) were added. After incubation at room temperature for 30 min, the solution in the tube was transferred to another tube to stop the labeling process. Radioiodinated LDL was separated from free ^{123}I on a 10 \times 1 cm Sephadex G25 column.

Iodination using N-bromosuccinimide (NBS method) (13). In this procedure, 6 μ l of a NBS solution (1 mg/ml 0.25 sodium phosphate buffer, pH 7.6) was added to a mixture of 0.5 ml LDL (1.46–3.68 mg protein/ml) and 0.5 mCi Na^{123}I . This mixture was allowed to stand at room temperature for 10 min. Then 0.5 ml of potassium iodide (1 g/ml) was added. The iodinated LDL was separated from free ^{123}I using a 10 \times 1 cm Sephadex G25 column.

Labeling yield and specific activity

Labeling yield was measured by determining the TCA (final concentration 10% w/v) precipitable radioactivity in the reaction mixture, using albumin as a carrier (final concentration 1% w/v). Alternatively, the amount of radioactivity in the LDL fraction after Sephadex G25 separation was expressed as a percentage of the total amount of radioactivity applied to the column.

Specific activity of radiolabeled LDL was expressed as mCi/mg protein in the LDL fraction after Sephadex G25 separation.

Radiolabel/protein ratio

The number of ^{123}I and $^{99\text{m}}\text{Tc}$ atoms incorporated in each LDL particle was calculated as follows:

$$N_{\text{RN}} = A_{\text{RN}} \cdot \frac{T_{\frac{1}{2}\text{RN}}}{\ln 2}$$

where N_{RN} is the number of radionuclide atoms, A_{RN} is the radioactivity of the radiolabeled LDL, and $T_{\frac{1}{2}\text{RN}}$ is half-life of the radionuclides. N_{RN} was then divided by the number of LDL particles, as calculated from the protein content of the radiolabeled LDL using 512 kD as molecular mass for apoB and assuming one apoB/LDL particle.

Total number of incorporated atoms/protein ratio

In addition to the radioactive ^{123}I and $^{99\text{m}}\text{Tc}$ atoms, nonradioactive ^{127}I and ^{99}Tc atoms are incorporated in LDL during radiolabeling. To calculate the total number of atoms incorporated in LDL, the following method was used:

$$\text{incorporated atoms per LDL particle} = \frac{\text{specific activity of labeled LDL}}{\text{specific activity of radionuclide solution}}$$

The specific activity of the ^{123}I solution used in the Iodogen and NBS method was 5 \times 10⁵ Ci/mol as supplied by the manufacturer. For the ICl method, the radioisotope solution was diluted with cold I during the labeling process, giving a specific activity of 3 \times 10⁴ Ci/mol.

For the $^{99\text{m}}\text{Tc}$ solution eluted from the $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ radionuclide generator, the specific activity was calculated using the following method. Since at the time of elution the generator is in equilibrium, the radioactivity of the Tc

solution (A_{Tc}) is equal to that of the ^{99m}Mo (A_{Mo}) at time of elution, and can be calculated using the equation:

$$A_{Tc} = A_{Mo} \cdot e^{-\lambda(Mo) \cdot T}$$

in which A_{Mo} is the radioactivity of the generator immediately after the previous elution of $^{99m}TcO_4^-$ expressed in Bq and λ_{Mo} is $\frac{\ln 2}{t_{1/2}}$ expressed in s^{-1} .

$$t_{1/2} \text{ (Mo)}$$

The total amount of Tc atoms in the elution equals the number of ^{99m}Mo atoms decayed since the previous elution 24 h before, and can be determined as follows:

$$^{99m}Tc \text{ atoms formed} = \int_0^{24} A_{Mo} \cdot e^{-\lambda(Mo) \cdot t} dt$$

Conversion to nmoles was performed by division of the number of Tc atoms by Avogadro's number.

Lipid-associated radioactivity

The amount of radioactivity bound to the lipid fraction of the LDL was determined for each of the labeling techniques after lipid extraction by the method of Bligh and Dyer (17). A mixture of 0.4 ml labeled LDL, 1.5 ml methanol-chloroform 2:1 (by volume), 0.5 ml chloroform, and 0.5 ml distilled water was vortexed and centrifuged for 10 min at 3000 rpm. The radioactivity in the chloroform layer was counted and expressed as percentage of the total radioactivity in the LDL sample.

Stability of the radiolabeled LDL

In order to investigate the stability of the association between the radiolabel and LDL, 30 μ l LDL labeled by each of the above methods was either *i*) mixed with 1 ml of unlabeled LDL; *ii*) mixed with 1 ml EDTA plasma; or *iii*) mixed with 1 ml of EDTA plasma and incubated for 15 h at 37°C. After this, gel permeation chromatography of each mixture was performed on a 80 \times 1.6 cm Sepharose CL6B column (20 mM HEPES-buffered saline, pH 7.4, as eluant). Fractions eluting at positions corresponding with those of VLDL, LDL, HDL + albumin, and low molecular weight material were identified by measurement of total cholesterol and triglycerides (Monotest® resp. Peridochrom®, Boehringer Mannheim, West Germany) and absorbance at 280 nm.

The radioactivities in these fractions were expressed as a percentage of total applied radioactivity. This permits a quantification of a possible shift of radiolabel to molecules other than LDL.

Agarose electrophoresis

To assess whether LDL was modified to a particle with an altered charge as a result of the different labeling procedures, agarose-gel electrophoresis of LDL labeled by the methods described above was performed to show possible changes in electrophoretic mobility. We labeled 2 mg LDL with either 8–11 mCi ^{99m}Tc for the ^{99m}Tc label-

ing methods or 0.5 mCi of ^{125}I for the radioiodination methods. For the electrophoresis we essentially followed the procedure as described by Demacker et al. (18), using a hippurate buffer (pH 8.8) instead of the barbiturate buffer (pH 8.8).

In vivo study

The clearance rate of ^{99m}Tc -labeled Fresh-LDL as produced by the dithionite method, the N_2S_2 method, and the borohydride method, was compared with that of ^{125}I -labeled Fresh-LDL labeled by the ICl method, which is the golden standard in LDL clearance studies. The same experiment was carried out using Pool-LDL, to compare the N_2S_2 method and the borohydride method with the ICl method. For this purpose 110–240 μ Ci of ^{99m}Tc -labeled LDL (1 mg apoB in 1 ml HBS, pH 7.4) was mixed with 50 μ Ci of ^{125}I -labeled LDL (1 mg apoB in 1 ml HBS, pH 7.4) as labeled by the ICl method, and the mixture was injected in two normolipidemic male NZW rabbits for each method. Blood samples of 1 ml were taken regularly and were counted for both radionuclides separately in a Scalar Ratemeter SR4 well-type gamma-counter. Values were corrected for physical decay of the radionuclides.

RESULTS

Labeling yield and specific activity

^{99m}Tc labeling methods. As shown in Table 1, the percentage of ^{99m}Tc that was incorporated in LDL differed widely among the four methods that we studied. Furthermore, the two tests used in this study to assess labeling yield (i.e., TCA precipitation and Sephadex G25 gel permeation chromatography) showed different efficiencies for the same radiolabeled LDL sample. The largest difference between the two tests was observed for the dithionite method (Pool-LDL): 70% when using TCA precipitation versus 13% after Sephadex G25. When Fresh-LDL was used, labeling efficiencies improved considerably in the dithionite method and DMF method, whereas only a minor increase in labeling yield was observed in the borohydride method. The labeling yield shown for the N_2S_2 technique represents the conjugation of the labeled active ester to the LDL. The efficiency of labeling the pentanoic acid compound itself was about 70%, resulting in an overall labeling efficiency between 50% and 65%.

^{125}I labeling methods. As in the ^{99m}Tc labeling methods above, a difference in measured labeling yield was observed when using Sephadex G25 gel permeation and TCA precipitation (Table 1). The difference was most pronounced in the NBS method (Fresh-LDL): 47% versus 91%, respectively. In all three methods, the use of Fresh-LDL showed only minor improvement in labeling efficiencies as compared with Pool-LDL.

TABLE 1. Results of radiolabeling of LDL by seven labeling methods

| Labeling Technique | n | Labeling Yield | | Specific Activity | Radionuclide/LDL Ratio | Total Incorporated Molecules/LDL Ratio | Lipid-Associated Radioactivity |
|--------------------------------------|-----|------------------------|--------------|-------------------|---------------------------------|--|--------------------------------|
| | | TCA Precipitation | PD 10 Column | | | | |
| | | % | | mCi/mg | | | % |
| Technetium | | | | | | | |
| Dithionite method | 4 | 84 ± 7 ^a | 61 ± 8 | 3.73 ± 0.24 | 3.7 ± 0.2 × 10 ⁻³ | 11.6 ± 0.1 × 10 ⁻³ | 4 ± 1 |
| | (3) | (70 ± 11) ^b | (13 ± 1) | (0.81 ± 0.08) | (8.0 ± 0.8 × 10 ⁻⁴) | (2.5 ± 0.5 × 10 ⁻³) | (5 ± 1) |
| DMF method | 3 | 78 ± 8 | 72 ± 5 | 4.40 ± 0.21 | 4.4 ± 0.1 × 10 ⁻³ | 13.9 ± 0.2 × 10 ⁻³ | 1 ± 1 |
| | (3) | (54 ± 8) | (30 ± 1) | (1.93 ± 0.06) | (1.9 ± 0.1 × 10 ⁻³) | (6.0 ± 0.1 × 10 ⁻³) | (2 ± 1) |
| N ₂ S ₂ method | 3 | 95 ± 3 ^c | 78 ± 4 | 3.11 ± 0.10 | 3.1 ± 0.1 × 10 ⁻³ | 9.7 ± 0.1 × 10 ⁻³ | 7 ± 3 |
| | (5) | (90 ± 2) ^c | (71 ± 5) | (2.92 ± 0.12) | (2.9 ± 0.2 × 10 ⁻³) | (9.1 ± 0.1 × 10 ⁻³) | (9 ± 2) |
| Borohydride method | 3 | 58 ± 5 | 50 ± 3 | 2.30 ± 0.11 | 2.3 ± 0.1 × 10 ⁻³ | 7.2 ± 0.1 × 10 ⁻³ | 3 ± 1 |
| | (3) | (56 ± 4) | (49 ± 3) | (2.23 ± 0.08) | (2.2 ± 0.2 × 10 ⁻³) | (6.9 ± 0.1 × 10 ⁻³) | (3 ± 2) |
| Iodine | | | | | | | |
| ICl method | 3 | 73 ± 7 | 58 ± 3 | 1.10 ± 0.09 | 1.4 ± 0.1 | 18.2 ± 0.4 | 4 ± 1 |
| | (3) | (70 ± 8) | (56 ± 5) | (0.76 ± 0.30) | (1.0 ± 0.4) | (13.0 ± 1.6) | (3 ± 1) |
| Iodogen method | 3 | 87 ± 6 | 71 ± 7 | 0.71 ± 0.09 | 0.7 ± 0.0 | 0.7 ± 0.0 | 7 ± 4 |
| | (3) | (82 ± 2) | (61 ± 7) | (0.63 ± 0.09) | (0.7 ± 0.0) | (0.7 ± 0.0) | (7 ± 3) |
| NBS method | 3 | 91 ± 2 | 47 ± 4 | 0.40 ± 0.04 | 0.4 ± 0.0 | 0.4 ± 0.0 | 8 ± 6 |
| | (2) | (88 ± 1) | (43 ± 3) | (0.37 ± 0.01) | (0.4 ± 0.0) | (0.4 ± 0.0) | (13 ± 8) |

^aLDL from freshly prepared plasma, mean ± SEM.

^bLDL from frozen plasma (in parentheses), mean ± SEM.

^cEfficiency of binding of active ester to LDL. Efficiency of ester synthesis was 70%, resulting in an overall efficiency 50–65%.

All labeling methods for LDL evaluated in this study showed specific activities of the labeled LDL that were satisfactory (> 0.25 mCi/mg) for the use of this LDL in scintigraphy (Table 1). A specific activity below 0.25 mCi/mg protein for a typical 4-mg protein injection is considered impractical because this results in prolonged data-acquisition during scintigraphy.

Radionuclide/LDL particle ratio and total incorporated atoms/LDL particle ratio

For the ^{99m}Tc labeling methods, the number of ^{99m}Tc atoms incorporated per LDL particle was highest with the DMF method (Fresh-LDL) i.e., 4.4 × 10⁻³ (number ^{99m}Tc plus ⁹⁹Tc atoms incorporated/LDL particle was 13.9 × 10⁻³) and lowest in the dithionite method (Pool-LDL), 8.0 × 10⁻⁴ (2.5 × 10⁻³) (Table 1). For the radioiodination methods, the ICl method (Fresh-LDL) showed the highest incorporation of ¹²³I, namely 1.4 atoms/LDL particle. The number of ¹²³I plus ¹²⁷I atoms/LDL particle was 18.2. Incorporation of ¹²³I was lowest with the NBS method (both Pool-LDL and Fresh-LDL) i.e., 0.4 atoms/LDL particle. Since no cold I was added during the latter labeling process, the total number of I atoms incorporated/LDL particle has the same value.

Lipid-associated radioactivity

For the ^{99m}Tc labeling methods, the values for chloroform-extractable radioactivity ranged from 1% for the DMF method (Fresh-LDL) to 9% for the N₂S₂ method (Pool-LDL). Of the iodination methods, the ICl method

(Pool-LDL) yielded 3% of radioactivity associated with lipids, whereas with the NBS method (Pool-LDL) 13% of radioactivity appeared to be lipid-associated (Table 1).

Stability of radiolabeled LDL in plasma

^{99m}Tc labeling methods. *Dithionite method.* Part of the ^{99m}Tc dissociated from LDL upon mixing with plasma (from 74% of the radioactivity associated with the LDL region down to 66%) and shifted to the HDL + albumin region and to the low molecular weight region when Pool-LDL was used. This shift was more pronounced (LDL region only 40%) after 15 h incubation at 37°C (Table 2). However, when Fresh-LDL was labeled by this technique, only a small loss of ^{99m}Tc from the LDL region was observed; 80% was recovered in the LDL region after 15 h incubation.

DMF method. Mixing of labeled Pool-LDL with plasma resulted in a small shift of ^{99m}Tc from LDL (from 70% label in the LDL region down to 64%) towards the HDL + albumin region and the low molecular weight region. The ^{99m}Tc shift towards these regions was much larger (only 29% remained in the LDL region) after incubation (Table 2). Incubation of ^{99m}Tc-labeled Fresh-LDL showed similar results (a drop in LDL-associated label from 81% to 31%).

N₂S₂ method. When using Fresh-LDL, there was a minor dissociation of ^{99m}Tc from LDL upon mixing with plasma (from 95% to 93% LDL-associated label) to the HDL + albumin region and low molecular weight region which increased somewhat (87% in LDL region) upon in-

TABLE 2. Distribution of radioactivity after size exclusion chromatography on Sepharose CL 6B of radiolabeled LDL, after mixture with unlabeled LDL, mixture with plasma, and after incubation with plasma at 37°C for 15 h

| Labeling Technique | Applied Sample | VLDL Region | LDL Region | HDL + Albumin Region | Low Molecular Weight Region | Recovery Radioactivity |
|--------------------------------------|--|-----------------------------------|------------|----------------------|-----------------------------|------------------------|
| Dithionite method | ^{99m} Tc-LDL mixed with cold LDL | 1% ^a (4%) ^b | 89% (74%) | 2% (10%) | 1% (6%) | 93% (94%) |
| | ^{99m} Tc-LDL freshly mixed with plasma | 1% (3%) | 87% (66%) | 5% (16%) | 1% (11%) | 94% (96%) |
| | ^{99m} Tc-LDL mixed with plasma, incubated | 1% (1%) | 80% (40%) | 9% (25%) | 4% (26%) | 94% (92%) |
| DMF method | ^{99m} Tc-LDL mixed with cold LDL | 1% (5%) | 81% (70%) | 4% (8%) | 3% (4%) | 89% (87%) |
| | ^{99m} Tc-LDL freshly mixed with plasma | 1% (3%) | 77% (64%) | 4% (12%) | 5% (10%) | 87% (89%) |
| | ^{99m} Tc-LDL mixed with plasma, incubated | 1% (1%) | 31% (29%) | 3% (37%) | 14% (22%) | 89% (89%) |
| N ₂ S ₂ method | ^{99m} Tc-LDL mixed with cold LDL | 2% (2%) | 95% (88%) | 2% (10%) | 0% (0%) | 99% (100%) |
| | ^{99m} Tc-LDL freshly mixed with plasma | 2% (2%) | 93% (85%) | 3% (12%) | 1% (0%) | 99% (99%) |
| | ^{99m} Tc-LDL mixed with plasma, incubated | 2% (2%) | 87% (73%) | 6% (21%) | 3% (3%) | 98% (99%) |
| Borohydride method | ^{99m} Tc-LDL mixed with cold LDL | 2% (2%) | 89% (85%) | 1% (1%) | 1% (2%) | 93% (91%) |
| | ^{99m} Tc-LDL freshly mixed with plasma | 2% (2%) | 87% (81%) | 2% (4%) | 2% (4%) | 93% (91%) |
| | ^{99m} Tc-LDL mixed with plasma, incubated | 2% (2%) | 85% (70%) | 3% (7%) | 4% (14%) | 94% (93%) |
| ICl method | ¹²³ I-LDL mixed with cold LDL | 3% (3%) | 83% (82%) | 3% (4%) | 3% (3%) | 92% (92%) |
| | ¹²³ I-LDL freshly mixed with plasma | 4% (6%) | 83% (75%) | 3% (3%) | 3% (3%) | 92% (87%) |
| | ¹²³ I-LDL mixed with plasma, incubated | 5% (8%) | 76% (71%) | 4% (3%) | 5% (5%) | 89% (87%) |
| Iodogen method | ¹²³ I-LDL mixed with cold LDL | 3% (3%) | 64% (52%) | 8% (11%) | 12% (22%) | 87% (88%) |
| | ¹²³ I-LDL freshly mixed with plasma | 4% (4%) | 60% (46%) | 9% (12%) | 13% (25%) | 86% (87%) |
| | ¹²³ I-LDL mixed with plasma, incubated | 4% (4%) | 24% (19%) | 6% (6%) | 52% (59%) | 86% (88%) |
| NBS method | ¹²³ I-LDL mixed with cold LDL | 3% (3%) | 55% (40%) | 10% (14%) | 25% (34%) | 93% (91%) |
| | ¹²³ I-LDL freshly mixed with plasma | 3% (4%) | 52% (36%) | 11% (14%) | 26% (39%) | 92% (93%) |
| | ¹²³ I-LDL mixed with plasma, incubated | 3% (3%) | 28% (18%) | 5% (5%) | 55% (67%) | 91% (93%) |

Radioactivity in each region is mean of two observations in which individual values do not differ more than 6%.

^aLDL from a freshly prepared plasma.

^bLDL from frozen plasma (in parentheses).

cubation with plasma (Table 2). For Pool-LDL, the shift of ^{99m}Tc was more pronounced: LDL-associated label decreased from 88% to 73% after incubation.

¹²³I labeling methods. ICL method. The loss of ¹²³I from iodinated LDL upon incubation was only slightly less for Fresh-LDL (a decrease from 83% to 76% LDL-associated ¹²³I) as compared with Pool-LDL (from 82% to 71%) (Table 2).

Iodogen method. A considerable loss of the ¹²³I was observed after incubation with plasma from both Pool-LDL (from 52% label in the LDL region down to 19%) and Fresh-LDL (from 64% down to 24%), suggesting a loose association of ¹²³I with the LDL (Table 2).

NBS method. ¹²³I dissociated from Pool-LDL to a large extent upon incubation with plasma (from 40% LDL bound label down to 18%, Table 2). The same was true for Fresh-LDL (from 55% to 28%).

Agarose-gel electrophoresis

Agarose-gel electrophoresis of LDL, radiolabeled by any of the different procedures as described in the Methods section, failed to show an increase in mobility of the LDL. However, the mobility of ^{99m}Tc-labeled LDL as obtained by the DMF method increased as the ratio ^{99m}Tc/protein was increased. The same was found after using

higher ¹²³I/protein ratios in the Iodogen method. When higher amounts of the active ester of the N₂S₂ ligand were reacted with LDL, a clear increase in mobility was observed (data not shown).

In vivo study

Seven radiolabeled LDL preparations that proved to be stable upon incubation with plasma in vitro (namely Fresh-LDL labeled by the dithionite method, N₂S₂ method, borohydride method, and ICl method, and Pool-LDL labeled by the N₂S₂ method, borohydride method, and ICl method) were injected into rabbits to study their behavior in vivo. As can be seen in Fig. 1, the rate of clearance from the circulation is practically identical for Fresh- and Pool-LDL radiolabeled by the N₂S₂ method and the ICl method. However, when the amount of N₂S₂ ligand was used as originally specified by Fritzberg et al. (9), (i.e., five times higher than the amount we used), an increase in decay of ^{99m}Tc-labeled LDL was observed (data not shown). Fresh-LDL labeled by the dithionite method was cleared slightly faster than LDL iodinated by the ICl method. ^{99m}Tc-labeled Fresh-LDL and ^{99m}Tc-labeled Pool-LDL as obtained by the borohydride method were cleared from the circulation considerably faster than LDL labeled by the ICl method.

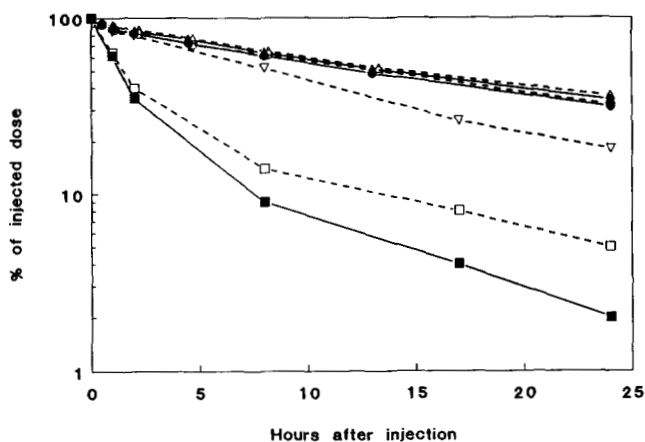


Fig. 1. Plasma decay curves in rabbits of LDL isolated from either fresh plasma (open symbols) or frozen plasma (closed symbols) radiolabeled by four different methods. Shown are: 1) ^{125}I -labeled LDL as produced by the iodine monochloride method (ICl method, Δ and \blacktriangle); 2) $^{99\text{m}}\text{Tc}$ -labeled LDL as obtained by a method using a diamide dithiolate derivative of pentanoic acid (N_2S_2 method, \circ and \bullet); 3) $^{99\text{m}}\text{Tc}$ -labeled LDL yielded by a method using sodium dithionite (dithionite method, ∇); and 4) $^{99\text{m}}\text{Tc}$ -labeled LDL produced by a method using sodium borohydride and stannous chloride (borohydride method, \square and \blacksquare). Values are mean of two observations; individual observations do not differ more than 4%.

DISCUSSION

Radiolabeled LDL may be a useful tool in noninvasive scintigraphic monitoring of the *in vivo* metabolism and biodistribution of LDL (1-4). In addition to this, the radiolabeled LDL may be applied in the diagnosis of atherosclerosis (4-7) because LDL accumulates in the atherosclerotic plaque by binding to matrix proteoglycans and by ingestion by macrophages present in the plaques (19-22). In this study we have focused on the application of radiolabeled LDL in biodistribution studies. For radiolabeled LDL to be a suitable scintigraphic agent in such studies, certain conditions must be fulfilled: the LDL should be labeled "hot" enough to provide a scintigraphic image of good quality within a suitable time period. Furthermore, the label should remain attached to the LDL particle after the radiolabeled LDL has been injected into the circulation. Finally, radiolabeled LDL should not be modified by the labeling procedure to the extent that its behavior in circulation on recognition by cellular receptors is different from that of unlabeled LDL.

It has been reported that loss of more than 15% of the ϵ -aminogroups of the lysine residues in the LDL protein leads to recognition by scavenger receptors and rapid uptake by the RES (23), resulting in an accelerated plasma clearance.

For many experiments, it is an attractive option to have a pool of LDL that can be used over a period of time in order to run different experiments using the same source of LDL. We therefore studied whether LDL isolated from

a pool of frozen plasma (Pool-LDL) gave labeling results similar to those observed using LDL obtained from freshly prepared unfrozen plasma (Fresh-LDL).

Each of the labeling methods showed sufficient labeling yield. Differences in labeling efficiencies as measured by TCA precipitation and Sephadex G25 chromatography might be partly explained by the observation that, in some labeling methods, e.g., in the dithionite method (Pool-LDL) and the DMF method (both LDL preparations), the radiolabel shifts from LDL to albumin as shown in Table 2. In TCA precipitation, albumin was added as a carrier and it might well be that a higher apparent labeling yield was the result of transfer of radiolabel to the albumin. On the other hand, less than total recovery of radiolabeled LDL from the Sephadex G25 column resulted in less than expected labeling yields. The use of TCA precipitation in $^{99\text{m}}\text{Tc}$ -labeling procedures can be questioned also for another reason, namely that the attachment of the $^{99\text{m}}\text{Tc}$ to the protein is not a result of the formation of a covalent bond, as is the case in the iodination procedures, but is caused by less strong associations that may be dissociated by the strongly acidic TCA. A high TCA precipitability indicates a strong binding of the radiolabel to the protein that is not disrupted by the TCA.

In the $^{99\text{m}}\text{Tc}$ -labeling methods, the use of Fresh-LDL showed an increase in labeling efficiency ranging from 2% (borohydride method, TCA) to as high as 140% (dithionite method, Sephadex G25) as compared with the labeling of Pool-LDL. For the iodination procedures this increase ranged from 3% (ICl method, TCA) to 10% (Iodogen method, Sephadex G25).

The stability of the radionuclide-LDL complex proved to be satisfactory only for the N_2S_2 method, the borohydride method, and the ICl method when Pool-LDL was used. When Fresh-LDL was used, the dithionite method also produced a stable radionuclide-LDL complex. Part of the radiolabel that did dissociate from LDL during incubation could have been lipid-associated and be exchanged to HDL.

Modification of LDL did not occur in any of the labeling methods as assessed by agarose-gel electrophoresis when radionuclide/LDL ratios were used as described in the Methods section.

No or minimal modification of $^{99\text{m}}\text{Tc}$ -labeled LDL was induced in our modification of the N_2S_2 method as was also suggested in the *in vivo* study, where it had practically the same rate of clearance from the circulation as ^{125}I -labeled LDL obtained by the ICl method, which is the standard for LDL labeling. However, our procedure differed from that described by Fritzberg et al. (9) in that we used five times less N_2S_2 ligand to react with protein. When the amount of ligand was used as proposed by Fritzberg, et al. (9), we observed increased clearance rates of the $^{99\text{m}}\text{Tc}$ -labeled LDL. $^{99\text{m}}\text{Tc}$ -labeled Fresh-LDL pro-

duced by the dithionite method was cleared at a slightly accelerated rate as compared with ^{123}I -labeled LDL obtained by the ICl method. The borohydride method yielded $^{99\text{m}}\text{Tc}$ -labeled LDL which was cleared from circulation at a considerably accelerated rate, regardless of whether Fresh-LDL or Pool-LDL was used. This implies that the borohydride method is not suitable for labeling LDL that is to be used in biodistribution studies.

In every labeling procedure that we studied, better labeling results were observed using Fresh-LDL instead of Pool-LDL. In pilot studies, we found that this was not caused by the difference in anticoagulation method (EDTA vs citrate). This observation suggests that, during storage of plasma at -80°C or due to the process of the freezing and thawing, the LDL is modified and that this modification interferes with the labeling procedures. The mechanism for the decrease in quality of the LDL is presently unclear.

In view of the altered labeling results of Pool-LDL as compared with Fresh-LDL, there is a possibility of further modifications of the Pool-LDL, not apparent in this study, that impairs its physiological behavior in vivo. This needs to be investigated in further studies. Therefore, it seems advisable to use only LDL isolated from freshly prepared plasma.

We conclude on the basis of these findings that only the N_2S_2 method, the dithionite method, and the ICl method yield radiolabeled LDL that can be used in biodistribution studies, provided that LDL isolated from freshly prepared plasma is used.

Whether any of the labeling methods is suitable for atherosclerosis localization studies needs to be further investigated. In such studies, modification and an accelerated plasma clearance of the radiolabeled LDL could be desirable properties, since modification of the LDL particle could lead to an enhanced uptake by the plaque-macrophages, and rapid plasma clearance would lower the background blood pool radioactivity, resulting in a better lesion-to-background ratio.

Since both $^{99\text{m}}\text{Tc}$ and ^{123}I are suitable radionuclides for imaging purposes, secondary aspects such as cost and availability play a role in the selection between them. In these respects $^{99\text{m}}\text{Tc}$ has distinct advantages over ^{123}I .

We conclude that 1) LDL that is to be used in biodistribution studies should preferably be isolated from freshly prepared plasma; and 2) as well as LDL labeled by the long-used ICl method, $^{99\text{m}}\text{Tc}$ -labeled LDL as obtained by the diamide dithiolate ligand method and $^{99\text{m}}\text{Tc}$ -labeled LDL produced by the dithionite method can be useful tools in the study of the transport and tissue localization of LDL in vivo in animals and man. ■

The authors gratefully acknowledge the labeling assistance provided by R. I. J. Feitsma and J. A. J. Camps. The authors also thank Dr. A. R. Fritzberg for his generous gift of the 4,5-

bis(benzoylthioacetamido)pentanoic acid compound. This work was supported by a grant from the Dutch Heart Foundation (project no. 87.042).

Manuscript received 4 December 1989 and in revised form 2 October 1990.

REFERENCES

1. Wynchank, S., J. E. Fincham, J. F. Klopper, H. J. Wasserman, and M. J. Weight. 1989. Biodistribution of ^{131}I -radiolabeled plasma low density lipoprotein in hyperlipidaemic vervet monkeys. *Atherosclerosis*. **80**: 159-167.
2. Rosen, J. M., S. P. Butler, G. E. Meinken, T. S. T. Wang, R. Ramakrishnan, S. C. Srivastava, P. O. Alderson, and H. N. Ginsberg. 1990. Indium-111-labeled LDL: a potential agent for imaging atherosclerotic disease and lipoprotein biodistribution. *J. Nucl. Med.* **31**: 343-350.
3. Sinzinger, H., J. Kaliman, H. Bergman, P. Angelberger, and R. Hofer. 1986. In vivo detection of increased ^{123}I -low-density-lipoprotein influx in human atherosclerotic lesions. Proceedings of 17th International Symposium on Radioactive Isotopes in Clinical Medicine and Research. **17**: 669-684.
4. Sinzinger, H., and P. Angelberger. 1988. Imaging human atherosclerosis with radiolabeled autologous low-density-lipoproteins (LDL) in human atherosclerosis. *Nucl. Med. Commun.* **9**: 859-866.
5. Lees, R. S., A. M. Lees, and H. W. Strauss. 1983. External imaging of human atherosclerosis. *J. Nucl. Med.* **24**: 154-156.
6. Lees, R. S., H. D. Garabedian, A. M. Lees, D. J. Schumacher, A. Miller, J. L. Isaacsohn, A. Derksen, and H. W. Strauss. 1985. Technetium-99m low density lipoproteins: preparation and biodistribution. *J. Nucl. Med.* **26**: 1056-1062.
7. Lees, A. M., R. S. Lees, F. J. Schoen, J. L. Isaacsohn, A. J. Fischman, K. A. McKusick, and H. W. Strauss. 1988. Imaging human atherosclerosis with $^{99\text{m}}\text{Tc}$ -labeled low density lipoprotein. *Arteriosclerosis*. **8**: 461-470.
8. Feitsma, R. J., D. Blok, M. N. J. M. Wasser, W. Nieuwenhuizen, and E. K. J. Pauwels. 1987. A new method for $^{99\text{m}}\text{Tc}$ -labeling of proteins with an application to clot detection with an antifibrin monoclonal antibody. *Nucl. Med. Commun.* **8**: 771-777.
9. Fritzberg, A. R., P. G. Abrams, P. L. Beaumier, K. Sudhakar, A. C. Morgan, T. N. Rao, T. M. Reno, J. A. Sanderson, A. Srinivasan, D. S. Wilbur, and J. L. Vanderheyden. 1988. Specific and stable labeling of antibodies with technetium-99m with a diamine dithiolate chelating agent. *Proc. Natl. Acad. Sci. USA*. **85**: 4025-4029.
10. McFarlane, A. S. 1958. Efficient trace-labeling of proteins with iodine. *Nature*. **182**: 53-57.
11. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoproteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta*. **260**: 212-221.
12. Fraker, P. J., and J. C. Speck, Jr. 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril. *Biochem. Biophys. Res. Commun.* **80**: 848-857.
13. Sinn, H. J., H. H. Schrenk, E. A. Friedrich, D. P. Via, and H. A. Dresel. 1988. Radioiodination of proteins and lipoproteins using N-bromosuccinimide as oxidizing agent. *Anal. Biochem.* **170**: 186-192.

14. Terpstra, A. H. M., C. J. H. Woodward, and F. J. Sanchez-Muniz. 1981. Improved techniques for the separation of serum lipoproteins by density gradient ultracentrifugation: visualization by prestaining and rapid separation of serum lipoproteins from small volumes of serum. *Anal. Biochem.* **111**: 149-157.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
16. Vallabhajosula, S., M. Paidi, J. J. Badimon, N. A. Le, S. J. Goldsmith, V. Fuster, and H. N. Ginsberg. 1988. Radiotracers for low density lipoprotein biodistribution studies in vivo: technetium-99m low density lipoprotein versus radioiodinated low density lipoprotein preparations. *J. Nucl. Med.* **29**: 1237-1245.
17. Bligh, E. G., and W. J. Dyer. 1959. A rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**: 911-917.
18. Demacker, P. N. M., H. E. Vos-Janssen, A. van 't Laar, and A. P. Jansen. 1978. A descriptive study of the different electrophoretic patterns on agarose of human serum very low density lipoproteins. *Clin. Chem.* **24**: 1439-1444.
19. Hoff, H. F., W. A. Bradley, C. L. Heideman, J. W. Gaubatz, M. D. Karagas, and A. M. Gotto. 1979. Characterization of low density lipoprotein-like particle in the human aorta from grossly normal and atherosclerotic regions. *Biochim. Biophys. Acta.* **573**: 361-374.
20. Linden, T., G. Bondjers, G. Camejo, R. Bergstrand, L. Wilhelmsen, and O. Wicklund. 1989. Affinity of LDL to a human proteoglycan among male survivors of myocardial infarction. *Eur. J. Clin. Invest.* **19**: 38-44.
21. Bocan, T. M. A., S. A. Brown, and J. R. Guyton. 1988. Human aortic fibrolipid lesions. Immunochemical localization of apolipoprotein B and apolipoprotein A. *Arteriosclerosis.* **8**: 499-508.
22. Daugherty, A. B., B. S. Sweifel, B. E. Sobel, and G. Schonfeld. 1988. Isolation of low density lipoprotein from atherosclerotic tissue of Watanabe heritable hyperlipidemic rabbits. *Arteriosclerosis.* **8**: 768-777.
23. Haberland, M. E., C. L. Olch, and A. M. Fogelman. 1984. Role of lysines in mediating interaction of modified low density lipoproteins with the scavenger receptor of human monocyte macrophages. *J. Biol. Chem.* **259**: 11305-11311.