Metabolism of Chromatographically Separated Rat Serum Lipoproteins Specifically Labeled with 125I-apolipoprotein E*

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Radioiodinated apolipoprotein E, added in small amounts to rat serum, rapidly associates with the serum very low density lipoproteins and high density lipoproteins in proportion to their content of endogenous apolipoprotein E. The labeled lipoproteins can be separated, without ultracentrifugation, by molecular sieve chromatography. When these labeled lipoproteins are injected intravenously into intact rats, the labeled apolipoprotein E rapidly exchanges with apolipoprotein E in the alternate lipoprotein fraction and is removed from the blood at a slow rate, comparable to that observed for apolipoprotein A-I in similarly labeled high density lipoproteins. No evidence was found for a rapidly turning over component of high density lipoproteins containing apolipoprotein E. When very low density lipoproteins, labeled endogenously with [3H]cholesterol and with radiiodinated apolipoprotein E, are added to perfusates of isolated rat livers, the labeled cholesteryl esters and apolipoprotein E are removed from the perfusate rapidly and at the same rate. Therefore, the slow removal of labeled apolipoprotein E in very low density lipoproteins in vivo is the result of rapid exchange with apolipoprotein E in high density lipoproteins.

Apolipoprotein E has been identified as a major protein component of remnants of triglyceride-rich lipoproteins in the rat (1), as well as the cholesterol-rich very low density lipoproteins and chylomicrons that characterize primary dysbetalipoproteinemia in humans (2) and the cholesterol-rich lipoproteins of cholesterol-fed mammals (3, 4). As the normally produced remnant lipoproteins are rapidly cleared by the liver (1, 5) and apo E in familial dysbetalipoproteinemia lacks isoforms normally present (6), it has been postulated that this protein may have a critical role in recognition of remnants by the liver (7).

Recent studies from several laboratories have provided evidence consistent with this hypothesis (8-17). Apo E is known to be synthesized in the rat liver (18-20) and is found in several plasma lipoprotein classes (4, 21-23). The metabolic behavior of apo E has been studied with radioiodinated lipoproteins in several laboratories (24-26). These studies have been difficult to interpret owing to rapid distribution of the radioiodinated apo E among lipoprotein classes and the ready dissociation of apo E from lipoproteins during isolation by ultracentrifugation (27, 28). By electrophoresis (29).

In previous studies from this laboratory, no detectable dissociation of apo E has been observed when lipoprotein fractions are separated from whole serum by gel chromatography (23, 27). Also, we have observed that apo E binds readily to chylomicrons (30). In the present research, we have specifically labeled lipoproteins in whole serum with 125I-apo E and have then separated the major labeled lipoproteins (VLDL and high density lipoproteins) by gel chromatography.

We report here some of the properties of this system of exogenous labeling and of the metabolism of the labeled lipoproteins.

** EXPERIMENTAL PROCEDURES **

** Materials**—[1,2-3H]Cholesterol, 40-60 Ci/mmol, was from New England Nuclear. Sodium (125)Ioside (carrier-free) was from Amersham/Shear.

Treatment of Rats—Male Sprague-Dawley rats weighing 300-350 g, or retired breeders from the same strain, maintained on standard Purina rat chow (Ralston Purina Co., St. Louis, MO) and tap water, were used. Blood was collected from the aorta of animals fasted for 24 h and anesthetized lightly with diethyl ether. The blood was kept on ice for 2 h and the serum was separated by low speed centrifugation at 4°C.

Isolation of Apoproteins—Rat apo E and apo A-I were isolated from pooled serum from retired breeders by conventional methods (22, 31). The purity of the apo E and apo A-I preparations was evaluated by isoelectric focusing (29) and sodium dodecyl sulfate-gel electrophoresis (32). The preparations were stored at -20°C and used within 3 months.

**Iodination of Apoproteins**—Iodination of apolipoproteins was carried out with the iodine monochloride method (33). Typically, 0.08-0.12 μg of protein in a volume of 0.1 ml was mixed with 20 μl of 1 M glycine-NaOH buffer (pH 10.0), 0.5-1.0 μCi of 125I, and 2 μl of ICl (4 × 10^-4 M in 2 M NaCl) at room temperature for 5 min. Unreacted 125I was removed by chromatography on Sephadex G-50 equilibrated with 2.5 M NaCl and 2 mM phosphate, pH 7.4, containing 5 μg/ml of bovine, fatty acid-poor albumin (Miles Laboratories, Inc., Kankakee, IL). More than 95% of the 125I was recovered from the column. A specific activity of 1000-2500 dpm 125I/μg of protein was routinely obtained (<0.25 μCi of iodine/100,000 molecular weight units of protein). Less than 1% of 125I associated with the protein was trichloroacetic acid-soluble (34). The 125I-labeled apoprotein was used immediately for incubation with serum.

** Incubation Procedure and Chromatographic Separation of Lipoproteins**—The 125I-labeled apoprotein fraction was mixed with 10 μl of serum and incubated for 1 h at 6 or 37°C with gentle shaking. One ml of 8% sucrose solution was added to the incubation medium and the sample was applied to a column (2.5 × 90 cm) of 6% agarose gel (1.5 M Bio-Gel, Bio-Rad, Richmond, CA), equilibrated with 0.11 mol of or 0.12 mol of NaCl and 0.01 mol of NaCl. The fractions were collected and assayed for 125I-specific radioactivity and anion-exchange chromatography (42). The various fractions were then subjected to isoelectric focusing (29) and sodium dodecyl sulfate-gel electrophoresis (32). The preparations were stored at -20°C and used within 3 months.
m NaCl and 2 mM phosphate, pH 7.4, containing 0.01% sodium azide. The flow rate was 15-20 ml/h and fractions of about 5 ml were collected. The column was operated at room temperature or at 6 °C. Columns operated under similar conditions exhibited comparable qualitative behavior. Fractions from the void volume, containing the VLDL fractions, were much smaller than 5 ml; the volume of each fraction was therefore measured. Absorbance of 280 nm and 205 nm were measured in each fraction.

Incubation Procedure with Isolated Lipoprotein Fractions—A portion (2.5 ml) of 125I-VLDL or 125I-HDL from the 6% agarose column was mixed with 10 ml of serum, obtained from fed animals. In this way less than 5% of the mass of cholesterol in the corresponding lipoprotein was added. The mixture was gently shaken at 37 °C for 30 min. When 125I-apo E-VLDL was incubated for 1 h (when 125I-apo E-HDL or 125I-apo A-I-HDL was incubated) the mixture was then chilled in ice and 1 ml of 80% sucrose was added. The sample was applied immediately to a 6% agarose column and eluted at 6 °C.

In Vivo Studies—The VLDL and HDL fractions from the 6% agarose column were dialyzed for 12 h against Krebs-Henseleit buffer (35) at 6 °C for 1-2 h. One ml of the radioiodinated lipoprotein, containing an amount of total cholesterol equivalent to less than 5% of that in the corresponding lipoprotein in the blood of the recipient rat, was injected through a femoral vein of fed animals. The flow rate was measured in each fraction. Absorbance of 280 nm and calculated as percentage of the injected 125I, assuming a plasma volume of 7.4 ml containing 18% rat erythrocytes in Krebs-Henseleit/glucose buffer, 3.9% water. Components were measured in individual fractions of the eluate. Recovery of apo E was 98% (mean of two experiments). Elution of 125I-apo E from 6% agarose gel columns was the same when serum was incubated with 125I-apo E at 37 or 6 °C for intervals of 1-24 h and when the columns were operated at 25 and 6 °C. When the pooled VLDL and pooled HDL fractions were rechromatographed on the same column of 6% agarose gel, all of the 125I-apo E remained with the VLDL or HDL; no unbound 125I-apo E was detected.

Perfusion of the Isolated Rat Liver—The VLDL used in these perfusions were labeled biologically with 3H in cholesteryl ester moiety (14) and with 125I-apo E, as described above, and diazoxid against Krebs-Henseleit buffer for 1-2 h. Albumin-dispersed [3H]-cholesterol (36) (250 µCi) was injected intravenously 8 h before the animals were bled from the abdominal aorta.

The general procedure for perfusion of the isolated rat liver has been described (34). Livers were first perfused with 30 ml of perfusate containing 15% rat erythrocytes in Krebs-Henseleit/glucose buffer, then flushed with 60 ml of perfusate and finally perfused in the recirculating system with 5 ml of perfusate to which the labeled lipoprotein had been added. One ml of sample was removed from the perfusate every 10 min. After removal of erythrocytes by centrifugation, trichloroacetic-soluble and -insoluble 125I were measured (34). The soluble fraction never exceeded 1% of the total 125I of the sample. At the end of the experiment as much blood as possible was obtained from the abdominal aorta, as described above. The liver was then removed and 4-6 samples from different parts of the liver were taken for analysis of 125I.

To determine trichloroacetic acid-soluble 125I in the total body, the rats were killed with diethyl ether. The skin including the tail and the feet were removed and the carcass was then homogenized in a Waring Blender in about 700 ml of acetone/alcohol (1:1, v/v). The volume of the mixture was measured and a portion was filtered. The filtrate was dried under N2 and dissolved in 0.15 m NaCl solution. Trichloroacetic-soluble 125I was assayed (34) and calculated as percentage of the injected 125I, assuming that the rat contained 80% water.

RESULTS

Chromatographic Separation of 125I-apo E Incubated with Serum—When serum from rats fasted for 24 h was separated on columns of 6% agarose gel, cholesterol eluted in three overlapping components, corresponding to VLDL, LDL, and HDL (Fig. 1). The HDL fraction overlapped the bulk of the serum proteins. Apo E eluted in two, well separated components, one coinciding with VLDL (11.9% of apo E applied (n = 2)), and the other with HDL (77.1%, n = 2); the latter extended somewhat into the LDL region. We have designated these fractions as VLDL and HDL. As reported previously (27), apo E in HDL was associated predominantly with larger HDL particles. No “free” apo E was detected.

The elution of 125I-apo E, incubated at 6 °C with serum for 1 h, from a 6% agarose gel column is shown in Fig. 2. Recovery of 125I-apo E was 92.4 ± 2.5% (n = 9), of which 14.2 ± 2.1% and 62.0 ± 3.1% were associated with VLDL and HDL, respectively. The remaining 16.2 ± 3.1% eluted in the region expected for unbound apo E. When 125I-apo E alone was applied to the column, it emerged in the same volume as the apparently unbound 125I-apo E fraction (not shown).

The pattern of elution of 125I-apo E from 6% agarose gel columns was the same when serum was incubated with 125I-apo E at 37 or 6 °C for intervals of 1-24 h and when the columns were operated at 25 and 6 °C. When the pooled VLDL and pooled HDL fractions were rechromatographed on the same column of 6% agarose gel, all of the 125I-apo E remained with the VLDL or HDL; no unbound 125I-apo E was detected.
To determine whether the $^{125}$I-apo E that eluted in the void volume of 6% agarose columns was in fact associated with VLDL, $^{125}$I-apo E VLDL from this column was separated on a column of 4% agarose gel. No $^{125}$I-apo E eluted in the void volume of the column; its pattern of elution corresponded to that of VLDL-cholesterol (Fig. 3).

Removal of $^{125}$I-apo E VLDL from the Blood—$^{125}$I-apo E VLDL, freshly eluted from columns of 6% agarose gel at 6 °C, as described above, were injected into intact rats. The $^{125}$I-apo E was removed from the blood slowly as compared with the removal of apo B of VLDL (40) (Fig. 4). However, when the $^{125}$I-apo E VLDL was 1) eluted from a 6% agarose gel column at room temperature, 2) stored for 24 h at 4 °C in column buffer, 3) dialyzed for 24 h against Krebs-Henseleit buffer, or 4) heated for 4 h at 37 °C, removal of the $^{125}$I-apo E VLDL from the blood was invariably rapid (Fig. 4) and more than 50% of the $^{125}$I was recovered in the liver 10 min after injection. Under all such conditions, the pattern of elution of the $^{125}$I-apo E VLDL from columns of 4% agarose gel was altered: a variable amount eluted in the void volume (presumably representing aggregated apo E) and the remainder eluted in the region expected for VLDL. However, when $^{125}$I-apo E was incubated with serum for 24 h rather than 1 h, and the labeled VLDL were then eluted from a 6% column of agarose gel at 6 °C, none of the $^{125}$I-apo E VLDL eluted in the void volume of 4% agarose gel columns and the $^{125}$I-apo E was slowly removed from the blood. In all subsequent experiments, $^{125}$I-apo E VLDL was eluted from 6% agarose gel columns at 6 °C and used within 4 h.

Chromatographic Separation of $^{125}$I-apo A-I HDL Incubated with Serum—In experiments similar to those described for $^{125}$I-apo E, $^{125}$I-apo A-I was incubated with rat serum. As shown in Fig. 4, the pattern of elution of $^{125}$I-apo A-I differed from that of $^{125}$I-apo E. No $^{125}$I-apo A-I was associated with VLDL or LDL. Except for a small amount of "free" apo A-I, all of the $^{125}$I-apo A-I was associated with HDL, present with smaller particles than those associated with $^{125}$I-apo E.

Removal of $^{125}$I-HDL from the Blood—When $^{125}$I-apo E HDL, eluted from columns of 6% agarose gel at 6 °C, were injected into fed rats, the labeled apo E was removed slowly from the blood (fractional catabolic rate was 11.2 ± 0.3% h⁻¹) (Fig. 4). This value is virtually identical to that reported by Sigurdsson et al. (41) of 11.9 ± 1.3% h⁻¹ for rat HDL isolated by sequential ultracentrifugation and then labeled with radiodiode by the iodine monochloride method. In such HDL, 60–65% of the radiodiode was in apo A-I.

Removal of $^{125}$I-apo E HDL from the blood was only slightly more rapid than that of $^{125}$I-apo A-I HDL (Fig. 6). No difference was observed in the removal of $^{125}$I-apo E HDL injected into fed animals or animals fasted for 24 h (not shown). One h after injection of the $^{125}$I-apo E HDL, 2.4 ± 0.8% (n = 4) of the $^{125}$I-apo E was associated with VLDL separated by chromatography on 6% agarose gel; the remainder was associated with HDL. In contrast, no $^{125}$I-apo A-I was found in VLDL at this time.

Transfer of $^{125}$I-apo E between VLDL and HDL—When $^{125}$I-apo E HDL was incubated for 1 h at 37 °C with whole serum, 9.2 ± 2.1% (n = 5) of the $^{125}$I-apo E was recovered in the VLDL fraction separated by chromatography (Fig. 7). This $^{125}$I-apo E VLDL eluted in the expected region from a...
Mass of apo E in VLDL or HDL, as determined by radioimmunoassay. This indicates that $^{125}\text{I}-\text{apo E}$ in VLDL exchanges with that in HDL. Such rapid exchange of $^{125}\text{I}-\text{apo E}$ between VLDL and HDL was only observed with freshly prepared, labeled VLDL. When $^{125}\text{I}-\text{apo E}$ VLDL had been kept overnight at 6 °C, it not only was rapidly taken up by the liver after injection into intact rats (Fig. 4), but no detectable $^{125}\text{I}$ was found in the HDL fraction separated by chromatography in comparable incubation experiments (not shown). This was also the case when the initial incubation of $^{125}\text{I}-\text{apo E}$ VLDL with serum was at room temperature, or when the labeled VLDL was incubated at 37 °C for 4 h or dialyzed overnight against Krebs-Henseleit buffer. The rapid exchange of freshly prepared $^{125}\text{I}-\text{apo E}$ VLDL with HDL evidently explains its kinetic behavior \textit{in vivo}. The rate of removal of $^{125}\text{I}-\text{apo E}$ VLDL and $^{125}\text{I}-\text{apo E}$ HDL from the blood during the first 60 min after injection of these labeled lipoproteins was virtually identical. After 1 h, 51.7 ± 3.5% ($n = 3$) and 49.8 ± 4.3% ($n = 3$) of the labeled VLDL and HDL, respectively, remained in the blood. Thirty min after injection of $^{125}\text{I}-\text{apo E}$ VLDL only 21.0 ± 7.1% ($n = 3$) of the $^{125}\text{I}$ apo E was associated with chromatographically separated VLDL; the remainder was associated with HDL (not shown).

\textbf{Uptake of $^3\text{H}$-Cholesteryl Ester and $^{125}\text{I}$-apo E VLDL by the Perfused Liver—VLDL labeled biologically with $^3\text{H}$-cholesterol esters and \textit{in vitro} with $^{125}\text{I}$-apo E as above, was added to liver perfusates and uptake of the isotopes was determined over short periods to minimize exchange of $^{125}\text{I}$-apo E with newly secreted lipoproteins. As shown in Fig. 9, the rate of removal of the labeled cholesteryl esters and apo E from the perfusate was similar, suggesting that $^{125}\text{I}$-apo E was taken up together with the VLDL particle. At the end of the 21 min experiment, 6.7% of the $^3\text{H}$-cholesterol esters and 15.4% of the $^{125}\text{I}$-apo E was associated with chromatographically separated perfusate HDL; the bulk of the labeled cholesteryl esters and labeled apo E remained with VLDL. At this time, 94.2% of the $^3\text{H}$-cholesterol esters and 89.7% of the $^{125}\text{I}$-apo E removed from the perfusate was recovered in the liver.

\textbf{Catabolism of $^{125}\text{I}$-apo E VLDL \textit{in Vivo}—Owing to exchange of $^{125}\text{I}$-apo E, the metabolic behavior of $^{125}\text{I}$-apo E VLDL does not provide evidence for a rapid uptake of apo E in association with VLDL, but the results of the liver perfusion experiments do. As some of the $^{125}\text{I}$-apo E injected into intact rats must be removed from the blood by the liver before exchange occurs with apo E in HDL, a fraction of the $^{125}\text{I}$-apo E injected into rats in VLDL should be rapidly catabolized to yield $^{125}\text{I}$-tyrosine and free $^{125}\text{I}$. By contrast, less $^{125}\text{I}$ injected
that retain their original complement of apo E. We and others was selected to permit metabolic studies with lipoproteins shown in Fig. (25, 26) obtained by chromatography on men-
tments; and dialyzed for 1-2 h against Krebs-Henseleit buffer. Rats were killed at the times indicated and the content of trichloroacetic acid-
soluble 125I in the carcass was determined as described under "Exper-
mental Procedures." Each point is the mean value for three experi-
ments; bars indicate S.D.

in HDL should be metabolized rapidly to such products. As shown in Fig. 10, the amount of trichloroacetic acid-soluble 125I increased more rapidly in the whole body of rats beginning 15 min after intravenous injection of 125I-apo E VLDL than after injection of 125I-apo E HDL.

**DISCUSSION**

The method of exogenous labeling used in the present study was selected to permit metabolic studies with lipoproteins that retain their original complement of apo E. We and others (25, 26) have observed that apo E generally is poorly labeled when isolated lipoproteins are radioiodinated. Separate labeling of the protein provided a means to label lipoproteins not only specifically, but with a specific activity sufficient for a variety of purposes. As the immediate objective was to study the metabolism of this apoprotein as a natural component of plasma lipoproteins, the observation that the distribution of the bound 125I-apo E closely followed that of the endogenous apo E in plasma lipoproteins during gel chromatography was encouraging. This observation indicates that the specific activity of apo E is similar among lipoprotein particles of very low as well as high density, as would be expected if 125I-apo E exchanged with unlabeled apo E in serum lipoproteins. Labeling by exchange is also consistent with the invariable presence of a small amount of unbound 125I-apo E. Shepherd and associates have previously shown that small amounts of added radioiodinated apo A-I exchange with unlabeled apo A-I in human HDL (42). However, it is not certain that exchange is the only mechanism of labeling. Addition of large amounts of apo A-I to human HDL produces an apo A-I-enriched particle (42). When lymph chylomicrons are exposed to apo E in serum (depleted of triglyceride-rich lipoproteins by ultracentrifugation), or to isolated apo E, the apo E content of the chylomicron particles increases many-fold. Under these conditions, the apo E displaces, at least partially, other surface components of the chylomicrons (phospholipids (30) or other apoproteins (16)). It must be kept in mind that such substitution can affect the metabolic behavior of the modified lipoproteins (16).

In the present experiments, transfer of 125I-apo E to lipopro-
teins was rapid and occurred readily at low temperatures, as found before for interaction of surface components of chylo-
microns with serum HDL (14, 30). The association of the bound 125I-apo E with both VLDL and HDL was stable upon rechromatography. Thus, this method appears to provide a means to obtain lipoproteins in which metabolically active surface components have not been detectably dissociated.

The 125I-apo E VLDL, freshly obtained from columns of 6% agarose gel, eluted from columns of 4% agarose gel with VLDL-cholesterol, indicating that the 125I-apo E eluted in the void volume of the 6% gel columns did not represent aggregated protein. However, with storage, even at 6 °C, the chromatographic behavior was altered, and a minor, but variable, amount of the labeled protein then eluted in the void volume of the 4% agarose column. Unlike VLDL, the labeled HDL appeared to be stable during storage for several days. The 125I-
apo E appeared to associate with HDL of larger than average size, whereas 125I-apo A-I, added similarly to serum, associated mainly with smaller HDL. This pattern closely resembles that for the endogenous apo E and apo A-I of rat HDL, as described previously (27) and confirmed here. Upon addition to serum, 125I-apo E in both the HDL and freshly prepared VLDL transferred readily from one lipoprotein class to the other. As this transfer was not accompanied by detectable change in the mass of apo E in the chromatographically separated lipopro-
tein classes, the movement of 125I-apo E appears to reflect molecular exchange. Such movement of labeled apo E has been observed by others (24) and evidently has a major influence upon the behavior of the labeled apo E injected intravenously into intact animals. Thus, we observed that 125I-
apo E, injected in either VLDL or HDL, was removed slowly from blood plasma; in both cases, most of the protein-bound 125I was found mainly in HDL separated from the blood serum by gel chromatography. We found no evidence for rapid metabolism of a component of HDL containing apo E, as suggested by observations with HDL separated by ultracentrifugation and heparin-affinity chromatography (13). If a fraction of the 125I-apo E in our HDL was metabolized rapidly,
the amount must be very small as no initial loss of a fraction of the injected apo E could be detected. The early phase of the removal of $^{125}$I-HDL reflects in part the transfer of the lipoprotein to extravascular fluid compartments. However, even though the apo E was associated with larger particles than apo A-I, $^{125}$I-apo E in HDL was removed from the blood slightly more rapidly than $^{125}$I-apo A-I in comparably labeled HDL. Furthermore, $^{125}$I-apo E injected in VLDL was converted to trichloroacetic acid-soluble products more rapidly than when injected in HDL (Fig. 10). These observations are consistent with the observed exchange of $^{125}$I-apo E in HDL with unlabeled apo E in VLDL (or VLDL remnants) which are known to be rapidly taken up and catabolized in the liver (14, 40, 43). Such rapid uptake of $^{125}$I-apo E in VLDL was in fact observed in the isolated, perfused liver, in which there was little or no opportunity for exchange of the labeled protein with unlabeled apo E in HDL (Fig. 9). Under this circumstance, apo E and VLDL-cholesteryl esters were taken up by the liver at comparable rates, consistent with endocytosis, as suggested from previous studies with plasma VLDL (40, 43) and VLDL isolated from perfusates of rat liver (14).

Rapid catabolism of apo E was also observed for $^{125}$I-apo E in aged VLDL, in which the pattern of elution from agarose gel columns was partially altered. The drastically altered behavior of the labeled apo E in vivo is evidently the result of the loss of its capacity to exchange with endogenous apo E in HDL. This raises the possibility that apo E in such VLDL may exist in more than one state. A functional significance for such “pools” of apo E remains to be demonstrated. However, other studies indicate that the affinity of apo E-containing lipoproteins for hepatic removal mechanisms (presumably receptor-dependent) differs (15, 16); it is therefore possible that the conformation of apo E at the particle surface influences lipoprotein metabolism.

In conclusion, the present results suggest that apo E remains with VLDL remnants as they are rapidly taken up by the rat liver. By contrast HDL containing apo E in chow-fed rats are not readily recognized by an hepatic receptor but are metabolized slowly by as yet undefined mechanisms.

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