In vivo conversion of human HDL_3 to HDL_2 and apoE-rich HDL_1 in the rat: effects of lipid transfer protein

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Abstract In this study we determined in vivo conversions of human ^{3}H-labeled cholesteryl ester-labeled HDL_3 ([^{3}H]CE-HDL_3) in male rats and the effects of partially purified lipid transfer protein on the conversion processes. Zonal centrifugation techniques were used to prepare the [^{3}H]CE-HDL_3 and to follow the conversion processes. One hour after the injection, a complete conversion of HDL_3 to and HDL_2-density species was found. With time, [^{3}H]CE separated with apoE-rich HDL_1 and, by 18 hr, 35.9% of plasma radioactivity was associated with the apoE-rich HDL_1 lipoprotein fraction. In vitro incubation of [^{3}H]CE-HDL_3 in rat plasma reproduced in part the $HDL_3 \rightarrow HDL_2$ conversion, but no movement of radioactivity to HDL_1 was observed. Injection of the rats with partially purified lipid transfer proteins induced [^{3}H]CE exchange between lipoproteins. The conversion of HDL_3 to HDL_2, however, was minimally affected. Formation of [^{3}H]CE-HDL_1, in contrast, was reduced to about one-half of that found in control animals. It is concluded that in vivo conditions are necessary for conversions of HDL_3 (and HDL_2) to HDL_1, and that lipid transfer reactions delay this process. —**Gavish, D., Y. Oschry, and S. Eisenberg.** In vivo conversion of human HDL_3 to HDL_2 and apoE-rich HDL_1 in the rat: effects of lipid transfer protein. *J. Lipid Res.* 1987. **28:** 257–267.

Supplementary key words cholesteryl esters • lecithin:cholesterol acyltransferase • low density lipoprotein

In recent years it has become apparent that several metabolic reactions participate in processes that regulate HDL levels and subpopulation distribution (1). It has been suggested that supply of free cholesterol and phospholipid (from lipolyzed and intact lipoproteins and from cell membranes) followed by cholesterol esterification causes conversion of small-sized and heavy HDL particles (e.g., HDL_3), to large-sized and less dense particles (e.g., HDL_2) (2–5). Another set of metabolic reactions appears to cause "reverse conversion" of HDL, i.e., formation of HDL_3 from HDL_2 (1, 2, 6, 7). Activity of the plasma lipid transfer proteins (8–14) seems to be essential for the "reverse conversion" reaction, possibly acting in concert with the hepatic lipase (1, 6, 7, 15, 16).

More recently, it has been proposed that the apoE-rich HDL_1 population, an HDL present normally in rats (17–20), also participates in the HDL conversion–"reverse conversion" reactions (1). According to this view, excessive accumulation of cholesteryl esters in HDL_2 (21), or perhaps even HDL_3 (22), results in conversion of apoA-I-rich HDL_2 (or HDL_3) to apoE-rich HDL_1. Whether lipid transfer proteins cause a "reverse conversion" of HDL_1 is not known.

HDL conversions and "reverse conversions" can be conveniently studied in the rat, an animal species whose plasma lacks lipid transfer activity (10, 19). When injected with [^{3}H]cholesteryl ester-labeled lipoproteins, movement of the radioactive molecule between HDL populations would indicate conversion of the whole lipoprotein particle. When such animals are treated with lipid transfer proteins, effects of "reverse conversion" could presumably be identified. These considerations led us to initiate an investigation of human HDL_3 conversions in rats and the effects of injected lipid transfer proteins on the conversion process. Because the injected HDL_3 was devoid of apoE, the investigation was particularly relevant to events that regulate the formation of apoE-rich HDL_1. The study indeed demonstrated that HDL_3 is a precursor of HDL_2 and of apoE-rich HDL_1, and that lipid transfer proteins delay the conversion process, especially to HDL_1.

METHODS

Preparation of high density lipoproteins

Human plasma was obtained from healthy normal volunteers after a 12-hr fast. Informed consent was ob-

Abbreviations: CE, cholesteryl ester; HDL, high density lipoprotein; LTP, lipid transfer protein; LCAT, lecithin:cholesterol acyltransferase; FC, free cholesterol.

tained from all subjects. The blood was collected in plastic tubes containing disodium EDTA (1 mg/ml), and the plasma was separated promptly at 4°C by low speed centrifugation (3000 rpm, 20 min). The plasma density was adjusted to d 1.063 g/ml with solid KBr, and the VLDL and LDL were removed after 18 hr centrifugation in a 60Ti rotor at 50,000 rpm at 4°C. The total HDL (d 1.063–1.21 g/ml) was then obtained from the infranatant by centrifugation at density of 1.21 g/ml for 48 hr at 50,000 rpm and 4°C. HDL_3 was prepared from total HDL by centrifugation in a zonal system (23), performed at 15°C in a Beckman Ti14 zonal rotor and a Beckman L5-50 ultracentrifuge. HDL_3 was separated at 41,000 rpm in a discontinuous NaBr gradient, spanning the density range 1.0–1.4 g/ml. Runs lasted exactly 22 hr. The rotor effluent was monitored by continuous measurement of absorbance at 280 nm by an ISCO model UA-5 Absorbance Monitor equipped with a quartz flow through cell. Fractions of 25 ml were collected. The HDL_3 fractions were identified, pooled, and dialyzed for 48 hr against several changes of 3 liters of 0.9% NaCl containing 0.01% EDTA (pH 7.6) and 0.01% NaN_3. Lipoproteins were concentrated to a volume of 3–6 ml by vacuum ultrafiltration (21). The chemical composition of zonally prepared human HDL_3 (mg/100 cholesteryl esters, 15.3 ± SD) was: protein, 55.0 ± 3.8; cholesteryl esters, 15.3 ± 2.3; triglycerides, 3.6 ± 1.3; free cholesterol, 1.6 ± 0.2; and phospholipids, 24.5 ± 2.1.

Preparation of human lipid transfer proteins

Lipid transfer proteins were purified by a modification of the method of Morton and Zilversmit (11) as described by Albers et al. (13) using phenyl-Sepharose and DEAE cellulose chromatography. After these two steps, the LTP is purified by 300- to 600-fold and is free of LCAT activity. LTP-containing fractions were identified, pooled, and concentrated by vacuum ultrafiltration. The concentrated LTP preparation contained about 1 mg of protein/ml and its activity was 5- to 10-fold higher than that of human d > 1.21 g/ml plasma fraction. LTP activity was assayed by the ability of the preparation to promote exchange of [^{3}H]CE between [^{3}H]CE-labeled human HDL_3 and unlabeled human plasma LDL. In the assay, 0.4 mg of cholesteryl ester of [^{3}H]CE-labeled HDL_3, prepared as described below, was incubated at 37°C in a shaking water bath with 2 mg of cholesteryl ester of unlabeled LDL. Incubations lasted 4 hr, and the LDL was separated from the HDL_3 by ultracentrifugation in a 40.3 rotor at 39,000 rpm, 4°, for 24 hr. Transfer activity was quantitated by the appearance of [^{3}H]CE in the LDL.

Preparation of [^{3}H]cholesteryl ester-labeled HDL_3

HDL_3 labeled with [^{3}H]CE was prepared by combination of the LCAT and LTP reactions as follows: 50 μCi of [7(n)-^{3}H]cholesterol (Amersham Radiochemical Centre, England) in 0.1 ml of ethanol solution was added to 10 ml of normal human plasma of d > 1.125 g/ml and incubated for 18 hr at 37°C. The density was then adjusted to 1.21 g/ml, and the plasma fraction of density 1.125–1.21 g/ml was obtained by centrifugation, as described above. More than 95% of the [^{3}H]cholesterol was in the form of [^{3}H]CE. Trace amounts of the [^{3}H]CE-HDL were incubated with 30–40 mg of native HDL_3 (prepared by zonal ultracentrifugation) and with 100 μl of partially purified human LTP at 37°C in a shaking water bath for 18 hr. At the end of the incubation, HDL was reisolated by zonal ultracentrifugation. The [^{3}H]CE-labeled HDL_3 thus prepared was homogeneous, eluted at the position of native HDL_3, and the radioactivity profile paralleled the protein profile **(Fig. 1).** This [^{3}H]CE-labeled HDL_3 had not been acted upon by LCAT, and contained negligible (unmeasurable) amounts of LTP. The chemical composition of the [^{3}H]CE-labeled HDL_3 was almost indistinguishable from that of the unlabeled HDL_3, the only exception being a slightly lower free cholesterol content, 0.9% of total mass.

Analytical methods

Lipoprotein protein was determined by the method of Lowry et al. (24) using bovine albumin as a reference

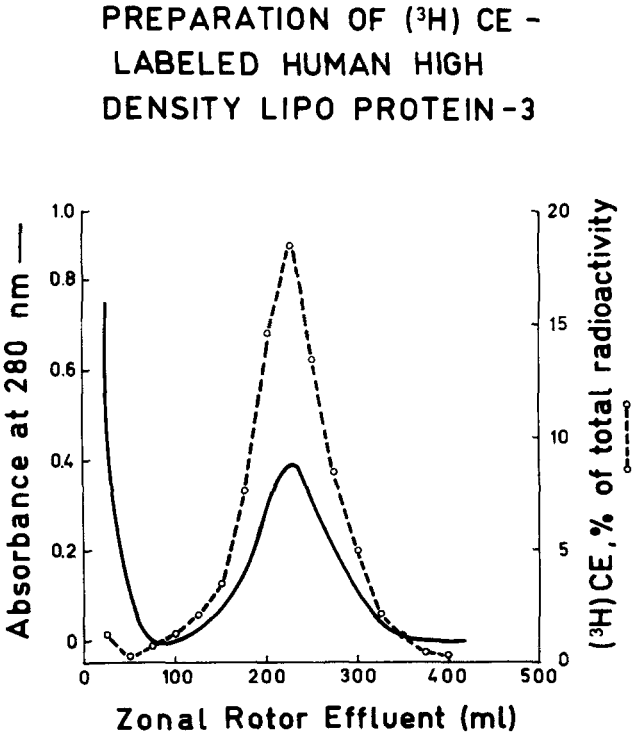

Fig. 1. Zonal elution profile of the protein mass (solid line) and the [^{3}H]cholesteryl esters (broken line) in [^{3}H]CE-labeled human HDL_3. The fraction that eluted between 175 and 300 ml was collected, concentrated, characterized, and used for further in vivo and in vitro experiments.

standard. Total lipoprotein cholesterol and total lipoprotein unesterified cholesterol were measured by cholesterol oxidase-cholesterol esterase procedure (25) using commercial kits (Boehringer, Mannheim, Germany). Lipoprotein cholesteryl ester content was calculated by difference. Apo-proteins were separated on an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system (26). Apoproteins were stained with Coomassie blue and identified by use of purified apoprotein standards for apoA-I, apoA-II, apoA-IV, apoB, apoC, and apoE. Lipids were separated by thin-layer chromatography in petroleum ether–ether–acetic acid 80:20:1 (v/v/v) as previously described (27).

HDL populations with and without apolipoprotein E were separated on heparin-Sepharose columns by the procedure of Weisgraber and Mahley (28) as described previously (29). With this procedure, an HDL devoid of apoE elutes first from the column, followed by apoE-containing HDL populations. SDS-PAGE analysis of the HDL fractions obtained using this procedure has recently been published (29).

Experimental procedures

Male rats of the Hebrew University strain (250–300 g) were used. [^{3}H]CE-HDL$_3$ was injected through an exposed saphenous vein to ether-anesthesized rats. Each rat received 2 mg protein, 1–2 $\times 10^5$ dpm of [^{3}H]CE-labeled HDL$_3$. Blood was collected at time intervals (2 min to 1080 min) from the abdominal aorta. The blood was allowed to clot on ice and serum was separated at 4°C by 20 min centrifugation at 3000 rpm. An 0.5-ml serum aliquot was taken for radioactivity determination and another 0.5 ml for thin-layer chromatography of lipids. Lipoproteins were separated by two methods: *1*) 5 ml of serum was taken for sequential separation of lipoproteins of d $<$1.006 g/ml, d 1.085 g/ml, d 1.125 g/ml, and d 1.21 g/ml in a 40.3 Ti rotor. Lipoproteins were isolated by the tube-slicing technique (30) and aliquots were taken to determine total fraction radioactivity and for separation of labeled lipid by thin-layer chromatography. Lipoprotein [^{3}H]CE plasma decay was determined as follows: Plasma radioactivity 2 min after the injection of the labeled lipoprotein was considered as the injected dose (100% radioactivity). Lipoprotein [^{3}H]CE as percent of injected dose was then obtained from percent radioactivity remaining in plasma at time intervals multiplied by the percent of [^{3}H]CE radioactivity in a lipoprotein fraction (d $<$1.006 g/ml = VLDL; d 1.006–1.085 g/ml = LDL + HDL$_1$; d 1.085–1.125 g/ml = HDL$_2$ and d $>$1.125 g/ml = HDL$_3$). *2*) Lipoproteins from rats injected with [^{3}H]CE-HDL$_3$ were also separated by centrifugation in a zonal system. To follow HDL$_3$ and HDL$_2$, plasma fractions of d $>$1.006 g/ml were applied to the bottom of a d 1.0–1.4 g/ml gradient and the HDL populations were separated after 22 hr centrifugation at 41,000 rpm (16, 21, 23). To follow [^{3}H]CE-labeled LDL, HDL$_1$, and HDL$_2$, a plasma fraction of d $<$1.21 g/ml was applied to the bottom of a 1.0–1.3 g/ml gradient and lipoproteins were separated after 170 min centrifugation at 42,000 rpm (19, 21). The rotor effluent was collected in 25-ml fractions and radioactivity was determined in each tube. [^{3}H]FC and [^{3}H]CE radioactivity in individual tubes or in pooled fractions were determined by thin-layer chromatography. The effects of LTP on HDL conversions were determined in groups of rats injected intravenously with 0.5-ml of concentrated, partially purified human LTP, 1 hr prior to administration of the [^{3}H]CE-HDL$_3$. The amount of lipid transfer activity present in the 0.5 ml LTP preparation was equivalent to that of 10 ml of normal human plasma. Distribution of radioactivity among lipoproteins in the LTP-injected rats was followed at time intervals by zonal ultracentrifugation as described above. The data obtained in these experiments were compared to those of control rats investigated simultaneously without LTP injection. The circulating half-life time of the injected human LTP in rats was determined in plasma samples obtained 2, 30, 120, 360, and 720 min after the LTP injection. Plasma LTP activity was determined by assays of transfer of [^{3}H]CE from human HDL$_3$ to LDL, as described above. In the assay, the activity of 0.5 ml of plasma obtained from rats injected with LTP was determined and compared to that of non-injected rats. Activity in the latter animals was indistinguishable from blank values (incubations of [^{3}H]CE-labeled HDL$_3$ and unlabeled LDL alone) and it increased by 4- to 6-fold in LTP-injected animals. The decay of LTP activity from the plasma was linear, and the half-life time of the injected LTP was 9–10 hr. Hence, approximately 40–50% of the activity found 2 min after the injection remained in plasma 10 hr later.

RESULTS

Separation of rat plasma lipoproteins

Elution profiles of rat plasma lipoproteins from the two zonal centrifugation systems (see Methods) are shown in **Fig. 2.** Panel A shows the profile obtained after a 22-hr HDL run. HDL$_2$ eluted as a symmetrical peak, with peak elution volume at 140 ml. Human HDL$_3$ eluted in this run with a peak at a rotor volume of 225 ml (see Fig. 1). Panel B presents separation of lipoprotein after a 170-min LDL run. With this procedure, LDL, HDL$_1$, and HDL$_2$ were separated (19, 21). With this run, human HDL$_3$ eluted with a peak at a volume of 525–550 ml.

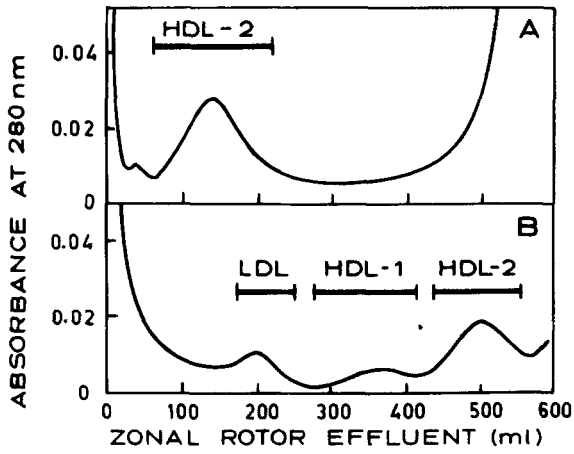

Fig. 2. Zonal elution profiles of rat plasma lipoproteins. Panel A shows the elution profile of rat HDL_2 after 22 hr centrifugation in a NaBr gradient of 1.0–1.4 g/ml, and panel B shows the elution profile of LDL, HDL_1, and HDL_2 after 170 min centrifugation in a NaBr gradient of 1.0–1.3 g/ml (see Methods).

Conversions of [^{3}H]CE-HDL_3 to HDL_2

To study conversion of [^{3}H]CE-labeled human HDL_3 in rats, animals were injected with 2 mg of HDL_3 protein and the elution profile of the radioactivity was followed by zonal ultracentrifugation **(Fig. 3).** Two minutes after the injection, the [^{3}H]CE eluted essentially at the same position as that of the injected HDL_3 (compare with Fig. 1). Thirty minutes later, most of the radioactivity eluted with HDL_2 (peak elution volume, 150 ml) with only small amounts at the original HDL_3 position. Sixty minutes after the injection, almost all of the plasma [^{3}H]CE was in HDL_2. Further movement of [^{3}H]CE to lipoproteins of light density was observed 6 hr after the injection when 20–25% of the plasma radioactivity was associated with the initial 50 ml of the zonal effluent. That movement of radioactivity to lighter lipoproteins was even more pronounced at 18 hr (1080 min) when about 50% of the plasma [^{3}H]CE was found in the first 50–100 ml of the zonal effluent. The elution profile of [^{3}H]CE was also determined after 1 hr incubation with 5 ml of freshly prepared rat plasma **(Fig. 4).** The 1-hr incubation resulted in a shift of density of the HDL_3 towards HDL_2 that was about half that found in vivo.

Conversion of [^{3}H]CE-HDL_3 to HDL_1

The movement of radioactive cholesteryl esters to lipoproteins of densities lighter than HDL_2 was investigated 3 to 18 hr after injection of 2 mg of protein of [^{3}H]CE-HDL_3 **(Fig. 5).** In these experiments, lipoproteins were separated using a NaBr gradient of 1.0–1.3 g/ml and 170 min centrifugation. Two minutes after the injection, the radioactivity separated exclusively in HDL_3, peak elution volume, 525–575 ml. At 180 min, the main radioactivity peak eluted at the position of HDL_2 (450–525 ml) with

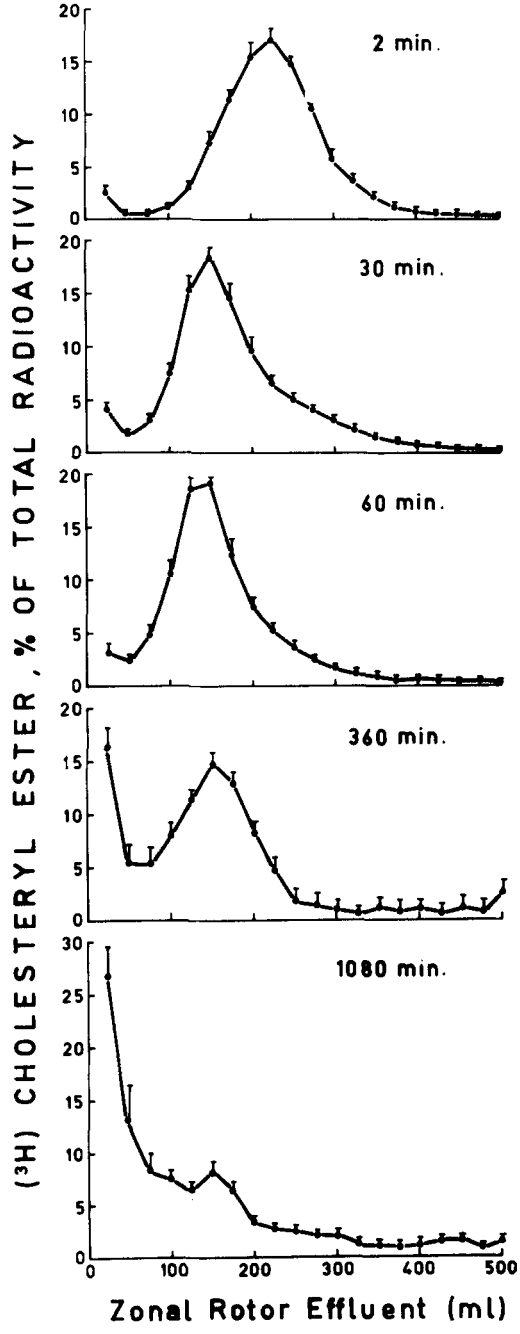

Fig. 3. Zonal elution profiles of [^{3}H]cholesteryl esters in HDL at time intervals after the injection of [^{3}H]CE-labeled human HDL_3 into rats. Lipoproteins were separated by centrifugation in a discontinuous NaBr gradient of density 1.0–1.4 g/ml and spun for 22 hr at 41,000 rpm. In this system, HDL_3 separated at effluent volumes of 150–350 ml (peak at 225–250 ml); HDL_2 at effluent volumes of 50–150 ml (peak at 120–140 ml); and lighter lipoproteins (VLDL, LDL, and HDL_1) at effluent volumes of less than 75 ml. Data are mean ± SEM of four to seven experiments.

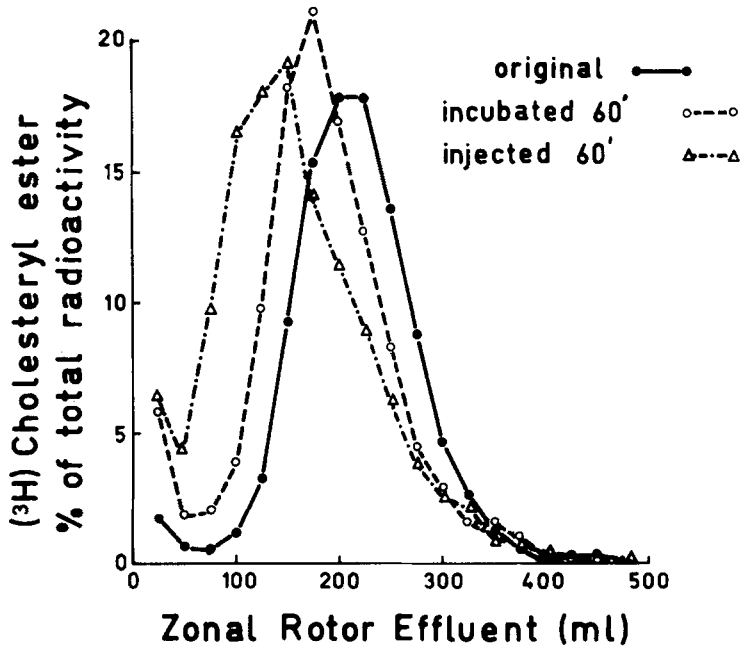

Fig. 4. The zonal elution profile of [^{3}H]CE-labeled human HDL_3 (●—●) as compared to the elution profiles of the same HDL following 60 min of in vitro incubation with rat plasma (○---○) or 60 min of in vivo interaction following its injection into rats (△·····△). Conditions of centrifugation are as described in the legend to Fig. 2. Data are from one representative experiment. Similar results were obtained in two other experiments.

minimal amounts of [^{3}H]CE in HDL_1 and LDL. Six hours after the injection, increasing amounts of [^{3}H]CE appeared with LDL (elution volume 175–250 ml) and HDL_1 (elution volume 275–425 ml) and peak radioactivity in HDL_2 was at a rotor volume of 450–475 ml. That transfer of radioactivity, especially to HDL_1, became very prominent 18 hr after the injection. At that time, 35.9% of plasma radioactivity was with HDL_1, compared with 8.8% with LDL. The elution profile of [^{3}H]CE after 18 hr of in vitro incubation of labeled HDL_3 with rat plasma was very different from that found in vivo **(Fig. 6).** In the incubated sample, a shift of density of HDL_3 towards HDL_2 was observed but almost no radioactivity was found in LDL and HDL_1.

Association of [^{3}H]CE with apoE in HDL_1

To ascertain that after the injection the [^{3}H]CE-HDL_3 had indeed formed HDL_1 and not merely changed density, HDL_1 was isolated from the plasma of rats 18 hr after the injection, and the association between [^{3}H]CE and apoE was investigated. The HDL_1 was prepared by centrifugation at density of 1.04–1.085 g/ml and was found to contain 39.6% of plasma radioactivity. Only 1.7% of the [^{3}H]CE was found in that fraction 2 min after the injection. The association between [^{3}H]CE and apoE in HDL_1 was determined by lipoprotein fractionation on heparin-Sepharose columns (28, 29). In the injected HDL_3, all the radioactivity eluted with the fraction of HDL that does not contain apoE (fraction 1, ref. 29) and none was found in fraction 2, where apoE-HDL_1 normally elutes. This observation supported the conclusion derived from SDS-PAGE analysis that the injected HDL_3 was devoid of apoE (see Methods). In HDL_1 isolated 18 hr after the injection, in contrast, 80–90% of the [^{3}H]CE eluted with the apoE-containing fraction (fraction 2 (29)) and only 10–20% with HDL devoid of apoE. SDS-PAGE confirmed the presence of apoE in fraction 2, and its absence from fraction 1, as previously reported (29).

Kinetics of [^{3}H]CE-HDL conversions

The kinetics of the conversion of [^{3}H]CE-labeled human HDL_3 in rats was investigated by using fixed angle centrifugation. For that analysis, it was assumed that most of the radioactivity in the plasma fraction of density 1.006–1.085 g/ml was associated with HDL_1 (designated 'HDL_1'), while that of density 1.085–1.125 g/ml was associated with HDL_2 (designated 'HDL_2'). Radioactivity found in the fraction of density greater than 1.125 g/ml was considered to represent predominantly the HDL_3 (designated 'HDL_3'). Two minutes after the injection, 75–80% of the radioactivity separated with 'HDL_3' and 20% with 'HDL_2' **(Fig. 7).** An average of 2.4% of the

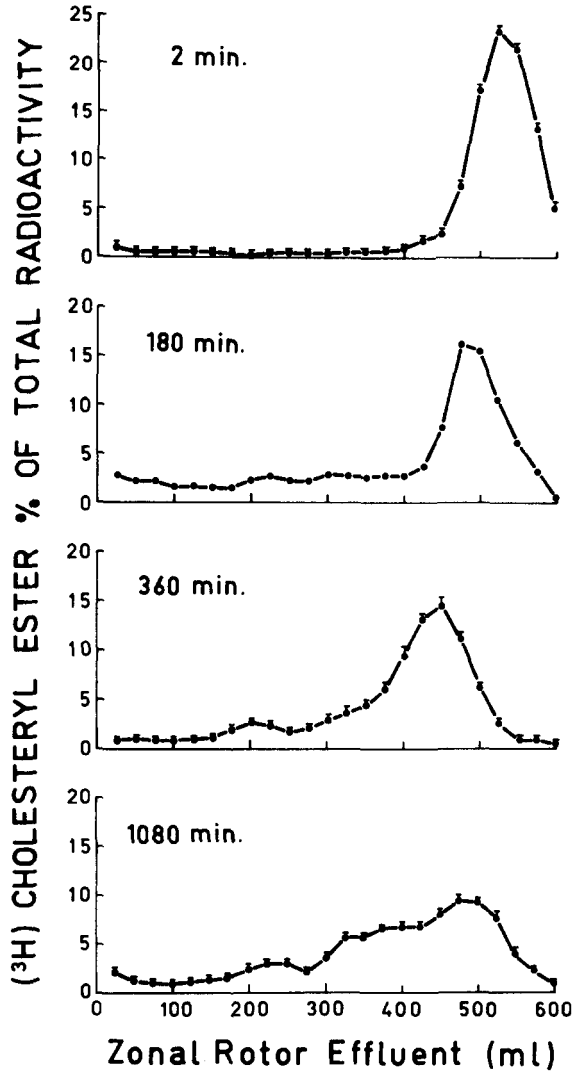

Fig. 5. Zonal elution profiles of [^{3}H]cholesteryl esters in low and high density lipoproteins at time intervals after the injection of [^{3}H]CE-labeled human HDL_3 into rats. Lipoproteins were separated on a linear 1.0–1.3 g/ml NaBr gradient after 170 min centrifugation at 42,000 rpm. After centrifugation, HDL_3 eluted at effluent volume of 475–600 ml (peak at 525–575 ml); HDL_2 at effluent volume of 425–550 ml (peak at 475–525 ml); HDL_1 at effluent volume of 275–425 ml (peak at 325–375 ml), LDL at effluent volume of 175–250 ml (peak at 225 ml), and VLDL at effluent volume of 0–75 ml. Data for the 2-, 360, and 1080-min intervals are the mean ± SEM of five experiments; data for the 180-min interval are the mean of two experiments.

radioactivity appeared in 'HDL_1'. Radioactivity disapeared rapidly from 'HDL_3' and accumulated in 'HDL_2', suggesting product-precursor relationship between the two. With time, a shift of radioactivity from 'HDL_2' to 'HDL_1' was found and by 12 hr 'HDL_1' contained more [^{3}H]CE than 'HDL_2'. The cross-over between 'HDL_1' and 'HDL_2' and the shape of the curves were compatible with conversion of part of the 'HDL_2' to 'HDL_1'.

Effects of injected lipid transfer proteins (LTP) on HDL conversions

To determine whether lipid transfer proteins affect the [^{3}H]CE-HDL conversions, some of the above experiments were repeated in rats that were injected with 0.5 ml of partially purified, concentrated, human LTP 1 hr prior to administration of [^{3}H]CE-HDL_3. In the first set of experiments, conversions of HDL_3 to HDL_2 were followed **(Fig. 8).** The movement of [^{3}H]CE from HDL_3 to HDL_2 in LTP-treated animals was slightly delayed (Fig. 8). Thirty minutes after the injection of [^{3}H]CE-HDL_3 to LTP-treated rats, less radioactivity was found in lighter fractions (volumes 50–150 ml) and more in heavier fractions (volumes 175–275 ml). These effects are best seen in the different plot (Fig. 8). Similar observations were made after 60 min. At both time intervals, however, most of [^{3}H]CE-HDL_3 moved to HDL_2. In the second set of experiments, movement of radioactivity to LDL and HDL_1 was followed **(Fig. 9).** LTP caused transfer of [^{3}H]CE to LDL and, in the treated animals, the LDL contained 50–100% more radioactivity than in the controls (18.3% and 9.0% of radioactivity, respectively, at 6 hr and $12.5 \pm 0.7\%$ and $8.2 \pm 1.1\%$ at 18 hr, $P < 0.05$). The behavior of HDL_1 was distinctly different. At the 6-hr time interval, HDL_1 in LTP-injected rats contained slightly more radioactivity than in the control animals. At 18 hr, however, considerably less radioactivity was found in HDL_1 of LTP-injected rats as compared to control animals, $25.4 \pm 2.8\%$ and $35.9 \pm 1.3\%$ of total [^{3}H]CE, respectively ($P < 0.01$). Accordingly, more radioactivity remained at the HDL_2 and HDL_3 positions. As in the previous set of experiments, these effects are best illustrated in the difference plots (Fig. 9).

DISCUSSION

The concepts of HDL conversion processes are based predominantly on in vitro experimental systems (1). The present investigation was undertaken to examine some of these concepts in an intact animal, the rat. In order to be able to follow HDL_3–HDL_2 conversions, and to use an HDL population without apoE, we decided to use human plasma HDL_3 labeled with [^{3}H]cholesteryl esters. The metabolic behavior of human HDL in rats has previously been reported to be almost indistinguishable from rat HDL when the apoA-I kinetics of the two were investigated (31, 32). As shown in the present study, the metabolic behavior of human [^{3}H]CE-labeled HDL in rats is also very similar to that found for rat HDL labeled biosynthetically with [^{3}H]cholesteryl esters (21).

The hypothesis that HDL_3 is a precursor of HDL_2 is based on in vitro experiments demonstrating a shift of

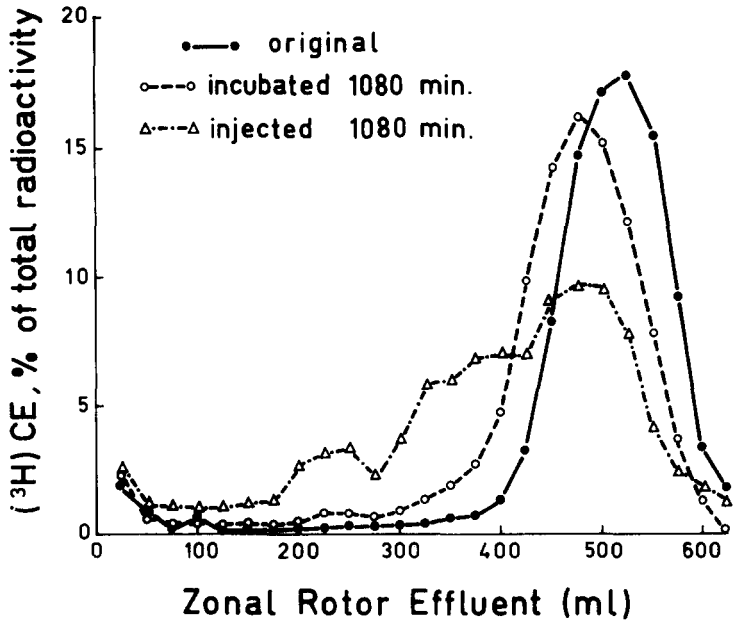

Fig. 6. The zonal elution profile of [^{3}H]CE-labeled human HDL_3 (●—●) as compared to the elution profiles of the same HDL following 1080 min of in vitro incubation with rat plasma (○---○) or 1080 min of in vivo interaction following its injection into rats (△-·-·-△). Conditions of centrifugation are as described in the legend to Fig. 5. Data are from one representative experiment. Similar results were obtained in two other experiments.

density of HDL_3 towards HDL_2 after assimilation of molecules originating from the surface coat of lipolyzed VLDL (33). This hypothesis is supported by reports that the variability of plasma HDL levels reflects variable HDL_2 concentrations while HDL_3 remains relatively constant (34). In the present investigation, conversion of HDL_3 to HDL_2 was demonstrated in vivo. We found that within 60 min of the injection of the rats with [^{3}H]CE-labeled HDL_3 almost all the radioactivity was associated with HDL_2. Kinetic analysis of this conversion indicated direct precursor-product relationship between the two that was practically complete. Naturally, in in vivo experiments it is impossible to determine which metabolic pathway is responsible for the $HDL_3 \rightarrow HDL_2$ conversion. Structural and compositional considerations demand that the HDL_3 doubles its core cholesteryl ester content and incorporates phospholipids, free cholesterol, and apoproteins into its surface coat (1). A factor in plasma that causes formation of HDL_2-like particles from HDL_3 after prolonged in vitro incubation has recently been described (35, 36). An incubation period of 12–24 hr with a rich source of "conversion factors" was necessary however to obtain partial conversion of pig HDL_3 to HDL_2 when LCAT was inhibited (36). In a similar study carried out by us with human HDL_3 (Gavish, D., Y. Oschry, and S. Eisenberg, unpublished observations), we also found that 12–24 hr of in vitro incubation periods with either human or rabbit lipoprotein-deficient sera (d $>$1.25 g/ml) are necessary for conversion of the HDL_3 to HDL_2, and that, even then, this process is incomplete. The in vivo conversion of HDL_3 to HDL_2 reported here, in contrast, was practically complete within 60 min (see Fig. 3). It therefore appears that while the activity of plasma conversion factors can be demonstrated in vitro, they are of no, or

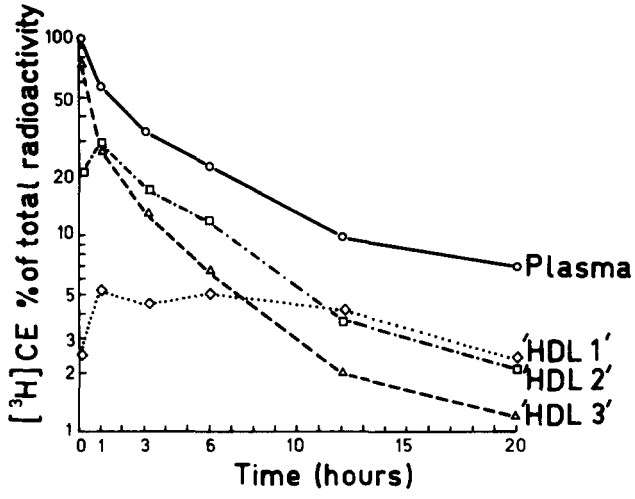

Fig. 7. Decay of plasma and lipoprotein [^{3}H]cholesteryl esters at time intervals after the injection of rats with 2 mg of protein of [^{3}H]CE-labeled human HDL_3. Lipoproteins were separated by sequential centrifugation as described in Methods. 'HDL_1', 'HDL_2', and 'HDL_3' are defined as the radioactivity in the plasma fractions of density intervals of 1.006–1.085 g/ml, 1.085–1.125 g/ml, and 1.125–1.21 g/ml, respectively. Data are means of three separate experiments.

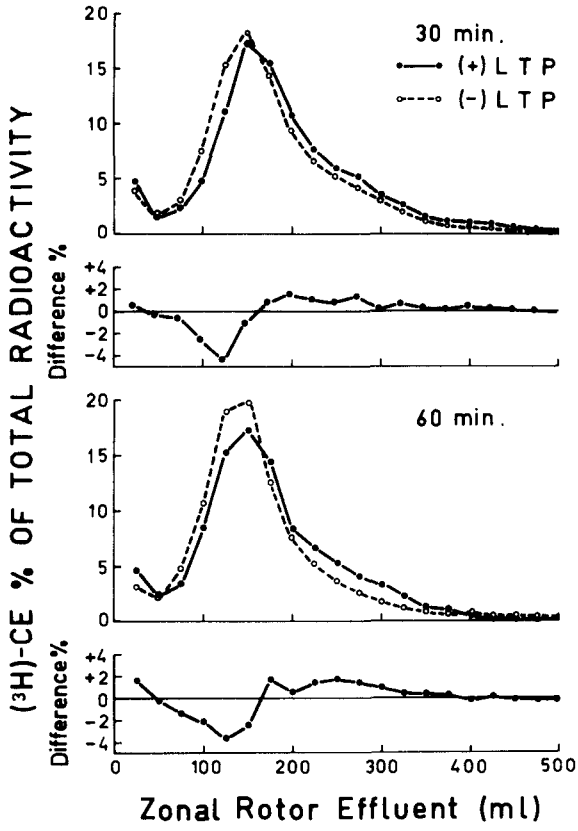

Fig. 8. Effects of injected lipid transfer proteins (LTP) on the HDL_3 to HDL_2 conversion process. Lipoproteins were separated in a zonal centrifugation system following the procedure described in the legend to Fig. 2. The difference plot represents data obtained in LTP-treated rats (+ LTP) minus the data from control animals (– LTP). Data are means of three separate experiments.

negligible, importance in vivo. These considerations led us to suggest that LCAT is the major enzymatic reaction responsible for the increase of HDL cholesteryl ester content in in vivo situations. The source of the surface constituents is presumably the outer coat of lipolyzed triglyceride-rich lipoproteins and molecules derived from cell membranes (1).

An apoE-rich HDL population, designated HDL_1, is a normal lipoprotein constituent present in rat plasma (17–20). In a previous experiment, we showed that part of the rat plasma HDL_2 is a precursor of HDL_1 (21). In that experiment, however, we could not rule out the possibility that the HDL_2 contained a subpopulation of particles rich with apoE that is destined to become HDL_1. The present study demonstrates that precursors devoid of apoE are transformed to particles of density of HDL_1. Of particular importance is the observation that the [^{3}H]CE found in HDL_1 is indeed associated with apoE, while no such association is found in the injected [^{3}H]CE-labeled HDL_3. Because conversion to HDL_1 does not occur after prolonged in vitro incubations, it is reasonable to assume that apoA-I exchange for apoE occurs in vivo and that apoE-rich HDL_1 is formed in plasma from precursors of HDL_3 and HDL_2 density. If that is the case, then apoE generated during lipolysis of triglyceride-rich lipoproteins is most probably the source of the apoE necessary for the apoprotein exchange. Indeed, lipolysis of triglyceride-rich lipoproteins is associated with transfer of apoE to particles of density and size similar to rat HDL_1 (37, 38). Important features of the HDL_1 conversion process are the inability to reproduce even part of this conversion in vitro and the relatively long period of time necessary for the conversion. Also, the kinetics of plasma [^{3}H]CE conversions indicate that only part of the HDL_2 radioactivity is moving to HDL_1. These observations further support our suggestion that LCAT rather than an HDL converting factor is responsible for the supply of cholesteryl ester molecules necessary for the formation of the larger and lighter HDL_1 from HDL_2. In contrast to the $HDL_3 \rightarrow HDL_2$ conversion, however, HDL_1 formation depends on activity of metabolic pathways that operate in vivo but are not reproduced during plasma incubations in vitro. Such pathways may include continued supply of

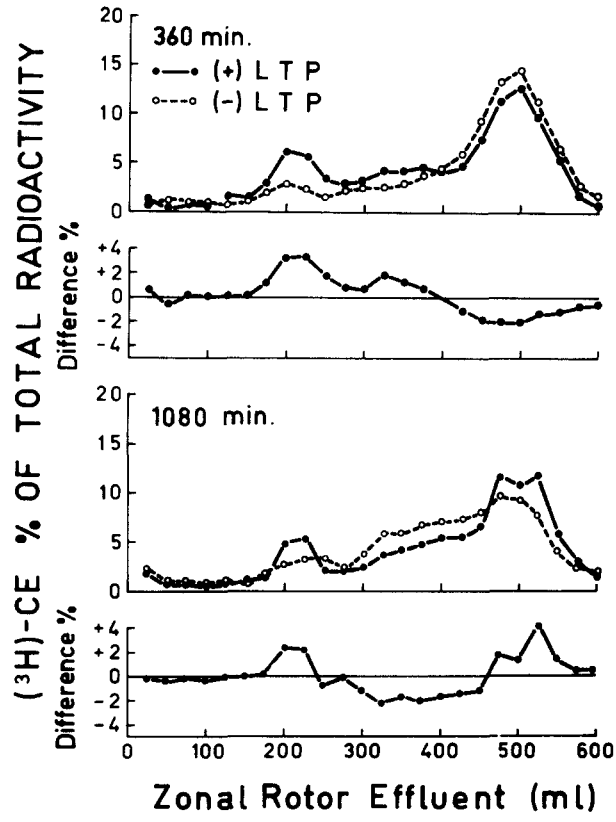

Fig. 9. Effects of injected lipid transfer proteins (LTP) on the conversion of HDL_3 to HDL_1. Lipoproteins were separated in a zonal centrifugation system following the procedure described in the legend to Fig. 5. The difference plot represents data obtained in LTP-treated rats (+ LTP) minus the data from control animals (– LTP). Data are means of three separate experiments.

free cholesterol and phospholipids from lipolysis and cell membranes for the LCAT reaction or interaction of the HDL_2 with cells or both. Formation of HDL_1 from HDL_3 has been previously demonstrated when the former lipoprotein is incubated in vitro with cholesterol-loaded macrophages under tissue culture conditions (22). Dependence of this process on supply of free cholesterol, apoE, and LCAT activity has been recently reported (39). Our study demonstrates formation of HDL_1 from HDL_3 in intact normal animals where, as discussed above, supply of cholesterol, phospholipids, and apoE is an integral part of the plasma fat transport system. It therefore appears that the plasma concentration of HDL_1-like particles will increase in situations where the supply of such constituents is increased. We suggest that cholesterol feeding and appearance of apoE-HDL_C(40) is an example when such a situation exists.

Using the rat model, it became possible to determine the potential effects of lipid (triglyceride and cholesteryl ester) transfer proteins on the two steps of HDL conversions discussed above. In agreement with a previous report (41), we found that the half-life of human LTP injected into rats is 9–10 hr (based on activity measurement). Hence, the activity of inhibitors to lipid transfer proteins described in rat plasma (42) was either insufficient or not effective in the system used by us. Judging from the ability to delay the movement of [^{3}H]CE from HDL_3 to HDL_2, lipid transfer proteins had only a modest effect on the $HDL_3 \rightarrow HDL_2$ conversion. We assume that that low magnitude delay of HDL_2 formation reflects the relatively slow effects of lipid transfer proteins on HDL (6, 7) in the face of a very rapid conversion process (60 min). The further conversion of HDL_2 to HDL_1, in contrast, was profoundly affected by the induction of lipid transfer reaction. Because cholesteryl ester exchange has been initiated in these animals, as evidenced by the increased amounts of [^{3}H]CE in VLDL, LDL, and HDL_1 at the 6-hr interval, it was expected to find even more [^{3}H]CE in these lipoproteins after 18 hr. Our data show that that indeed was found for LDL. HDL_1, however, contained substantially less [^{3}H]CE in rats injected with lipid transfer protein preparations as compared to intact animals. We estimate that in the injected rats the amount of HDL_1 formed was about one-half that of the control animals. Thus, regardless of the origin of apoE-rich HDL_1 (cells or intravascular events or both), circulating lipid transfer proteins appear to inhibit the formation of this lipoprotein.

In the present study, human HDL_3 was used and followed through density conversions in the rat. A change of the HDL apoprotein and surface lipid composition must have taken place during the conversion process. It is also possible that, in view of the known apoprotein heterogeneity of HDL populations (1, 33, 43), alterations of apoprotein profiles have occurred. Following density shifts, however, we cannot identify precisely the nature of the HDL_2 population formed in the rat from the injected human HDL_3, other than the change of density. In spite of this uncertainty, the experiments strongly suggest that the HDL system reflects equilibrium of several metabolic pathways that operate in vivo **(Fig. 10).** Conversion of the smallest HDLs (e.g., HDL_3 or HDL_4 (2)) to larger and lighter populations (e.g., HDL_2) occurs predominantly in the plasma compartment and depends on LCAT activity. When this process is rapid, "reverse conversions" (or delayed conversion) by the lipid transfer reaction is minimal. The effects of lipid transfer, however, become prominent when the conversion is slower or when acceptors for cholesteryl esters are present in excess, e.g., hypertriglyceridemia (44). The reactions that determine HDL_1 concentrations appear to be more complex. The conversion process itself ($HDL_2 \rightarrow HDL_1$ or $HDL_3 \rightarrow HDL_1$) seems to depend on conditions present only in the whole animal and operating for relatively long periods. It has been suggested that cells, in particular, macrophages, play an important role in processes leading to the formation of apoE-rich HDL_1(45). Our study neither rules out nor supports this notion. Yet, it is doubtful that cholesterol-loaded macrophages are present in large number in normal rats. Furthermore, the delay of HDL_1 conversion observed in lipid transfer protein-treated animals indicates that metabolic events affecting this lipoprotein occur in the vascular compartment. Thus, even though in vivo conditions are necessary, it appears that regulatory events affecting HDL_1 in either direction (conversion or "reverse conversion") operate in part or even in whole in or very close to the plasma compartment. That hypothesis, however, needs further evaluation.

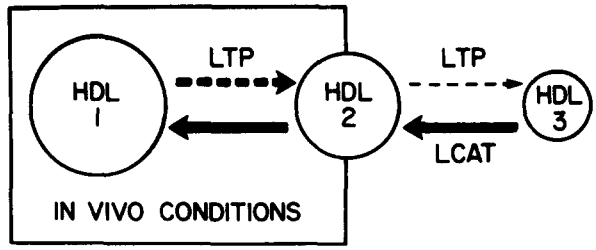

Fig. 10. Diagrammatic representation of HDL conversion and "reverse conversion" processes. Small HDL particles, represented by HDL_3, acquire cholesteryl esters in plasma through the activity of the LCAT reaction. This process requires relatively small numbers of cholesteryl ester molecules, occurs rapidly, and is therefore relatively resistant to "reverse conversion" by lipid transfer proteins (LTP). Further conversion of the HDL_2 to HDL_1 requires a considerable enrichment of the lipoprotein with cholesteryl esters and is observed only in vivo. The conversion of HDL_2 to HDL_1 is incomplete, occurs over a relatively long period of time, and is considerably more sensitive to the LTP reaction.

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