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High-density lipoprotein subpopulations as substrates for the transfer of cholesteryl esters to very-low-density lipoproteins

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INTRODUCTION

Both human HDL₃ and HDL₄ are heterogeneous and polydisperse lipoprotein groups which can be fractionated into several subpopulations with different particle masses, densities and compositions [1]. The existence of HDL particles with distinct apoprotein compositions (particles containing apo A-I with or without apo A-II and apo E [2–5]) makes the study of these subpopulations very important, since those apoproteins have a unique physiological significance [6]. On the other hand, this complexity hampers the interpretation of the results obtained with total HDL or even with HDL₃ and HDL₄ separately, as they contain particles with potentially different metabolisms.

HDLs prelabelled with radioactive cholesteryl esters are usually a prerequisite for the study of cholesteryl ester transfer protein (CETP) activity and other metabolic studies such as HDL–cell interactions. The correct interpretation of the results obtained with this kind of preparation essentially depends that HDL be uniformly labelled. Several approaches have been described for the labelling in vitro of these lipoproteins, either directly with radioactive cholesteryl esters [7–9], or with free cholesterol followed by incubation with active LCAT for cholesterol esterification [10–12], and the possibility exists that the labelling is not homogeneous, i.e. the different HDL particles incorporate the radioisotope with different efficiencies. In the present work we demonstrate in certain conditions that the labelling of human total HDL, HDL₃ or HDL₄ with [³H]cholesteryl oleate in the presence of lipoprotein-deficient serum (LPDS), as a source of lipid-transfer proteins, results in lipoprotein particles with highly different specific radioactivities which can be separated by heparin–Sepharose column chromatography. Moreover, these particles show different susceptibilities to precipitation by the reagent heparin–MnCl₂. The implication of this in the study of the CETP action is discussed.

MATERIALS AND METHODS

Isolation of plasma lipoproteins

VLDL (d < 1.006), HDL₃ (d = 1.063–1.215), HDL₄ (d = 1.063–1.125), HDL₃ (d = 1.125–1.21) and LPDS (d > 1.24) were separated by preparative sequential ultracentrifugation in a 50.2 Ti Beckman rotor [13] from pooled human normal lipoproteinemic sera, which contained Na₂EDTA (1 g/l), Trasylol (300 I.U./ml) and NaN₃ (0.2 g/l) as preservatives. To obtain rat LPDS, sera from several male Wistar rats were pooled, adjusted to d = 1.24 with solid KBr and ultracentrifuged as above. All the lipoproteins and the LPDS were exhaustively dialysed at 4 °C against 0.15 M NaCl/1 mm-Na₂EDTA, pH 7.4. Both human and rat LPDS were finally adjusted to the starting serum volume with the dialysis medium.

Labelling with [³H]cholesteryl oleate

Labelling of the HDL preparations with [³H]cholesteryl oleate was by the method of Roberts et al. [8]. In brief, approx. 25 μCi of [¹²⁵I,²⁵⁴C]cholesteryl oleate (65.8–82.9 Ci/mmol; New England Nuclear, Bad Homburg, Germany) in 0.1 ml of acetone was dripped over 15 ml of human LPDS with stirring, and the acetone was evaporated under a N₂ stream. A given volume of [³H]-containing LPDS was mixed with an equal volume of total

Abbreviations used: VLDL, very-low-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; apo, apoprotein; LPDS, lipoprotein-deficient serum; CETP, cholesteryl ester transfer protein; LCAT, lecinthin: cholesterol acyltransferase.

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HDL, HDL₂ or HDL₃ (2–5 mg of cholesterol/ml) and incubated at 4 °C for either 30 min or 17 h, or at 37 °C for 17 h. The density was increased with solid KBr, and the solutions were ultracentrifuged at 43000 rev./min for 40 h in a 45.6 TFT rotor (Kontron, Zurich, Switzerland) for the isolation of the [³H]cholesterol oleate-labelled HDL₄ (d = 1.125), HDL₅ or total HDL (d < 1.21). Radioactivity recovered in floated HDL was always over 61%. The [³H]-labelled HDLs were finally dialysed against 0.15 mM-NaCl/1 mM-Na₂EDTA, pH 7.4.

Labelling with [¹⁴C]cholesterol

Approx. 4 μCi of [⁴-¹⁴C]cholesterol (60.9 mCi/mmol; New England Nuclear) in 0.1 ml of ethanol was slowly injected into 15 ml of human LPDS with stirring and then mixed with an equal volume of HDL (2 mg of cholesterol/ml). The mixture was incubated for 18 h at 37 °C in a shaking bath to allow for cholesteryl ester formation by LCAT action [12]. The [¹⁴C]-labelled HDL was isolated by ultracentrifugation at d = 1.21 as described above and dialysed against 0.15 mM-NaCl/1 mM-Na₂EDTA, pH 7.4. Radioactivity recovery in the HDL fraction was over 76%.

HDL fractionation by heparin–Sepharose affinity chromatography

When further subfractionation by heparin–Sepharose chromatography was undertaken, the radioactively labelled HDL preparations were dialysed against 0.05 mM-NaCl/5 mMTris/HCl, pH 7.4, then supplemented with solid MnCl₂ to give a final concentration of 25 mM and loaded into chromatography columns containing heparin–Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) at approx. 1 mg of HDL-cholesterol/3 ml of gel [4]. After equilibration for 60 min at 4 °C, the samples were eluted at a flow rate of 24 ml/h with a discontinuous gradient of 5 mM-Tris/HCl, pH 7.4, containing 0.05 mM-NaCl/25 mM-MnCl₂ (solution A), 0.095 mM-NaCl (solution B) or 0.29 mM-NaCl (solution C). Fractions were collected and processed for measurement of A₂₆₀, radioactivity counting and determination of total cholesterol with a commercial enzymic kit (Menarini, Firenze, Italy). The fractions corresponding to the same peak were pooled, dialysed against 0.15 mM-NaCl/1 mM-Na₂EDTA, pH 7.4, and processed for determinations of protein [14] and of total (Menarini) and free (Biotrol, Louvres, France) cholesterol as well as radioactivity counting. The recovery of protein or [³H] radioactivity after chromatography was always greater than 86 and 78% of their respective amounts before dialysis. Lipids were extracted with chloroform/methanol (1:1, v/v) [15], and free and esterified cholesterol were separated by silicic acid t.l.c. with n-heptane/diethylether/acetic acid (75:25:4, by vol.) as solvent [16]. The spots were detected by exposure to iodine vapour, scraped off, and their radioactivity was counted. Radiolabelled lipoproteins were finally passed through 0.45 μm-pore-size filters and kept at 4 °C for up to 1 month without appreciable changes. Samples of the chromatographic fractions were delipidated with ethanol/acetone (1:1, v/v), resuspended in 8 mM-urea containing 10 mM-Tris/HCl (pH 8.6) and 10 mM-dithiothreitol and processed for gel isoelectrofocusing in the pH range 4–6.5 as described [17].

Incubation of [³H]-labelled HDL₂ and HDL₃ with LPDS

To study the transfer of [³H]cholesterol oleate to VLDL, 100 μl of prelabelled total HDL₄, total HDL₃ or their heparin–Sepharose chromatography fractions, containing 1.6, 6.8 or 15.4 μg of esterified cholesterol respectively, were mixed with 100 μl of VLDL suspension containing 0–60 μg of triacylglycerol in 0.15 mM-NaCl/1 mM-Na₂EDTA, pH 7.4, and 100 μl of human or rat LPDS. The tubes were incubated at 4, 25 or 37 °C for 0–120 min. To stop the reaction and to precipitate VLDL, 0.7 ml of human serum and 0.1 ml of a solution containing heparin (14.5 g/l) (from pig intestinal mucosa; Sigma Chemical Co., St. Louis, MO, U.S.A.) and MnCl₂ (1 mM) [18] were added to each tube. After mixing and standing for 10 min, the tubes were centrifuged at 1000 g for 30 min. Samples of the supernatants were used for [³H] counting, and the pellets were resuspended with 1 ml of human serum and precipitated as above. The washed pellets were resuspended with 0.5 ml of 0.9 % NaCl and transferred to scintillation vials. Recovery of the initial radioactivity in supernatant and pellet was always greater than 96% in any preparation. All radioactivity measurements were done with the OptiPhase HiSafe II scintillation cocktail (Pharmacia LKB Biotechnology) and a Beckman LS-3800 liquid-scintillation counter. Every experiment was repeated at least once under the same conditions but with different samples of [³H]-labelled HDL and VLDL, and the tubes were always run in duplicate. Results are expressed as percentages of the initial radioactivity precipitated and correspond to the means of the replicates. The transfer of [³H]cholesterol oleate from HDL to VLDL (expressed as μg of esterified cholesterol/2 h) was calculated as the difference between values obtained in the absence and the presence of VLDL in the media, the appropriate corrections being made for the HDL concentration used and the [³H]cholesterol oleate specific radioactivity in HDL.

To determine the mass of cholesterol precipitated by the heparin–Mn²⁺ reagent, different amounts of HDL-cholesterol (in 200 μl of 0.15 mM-NaCl/1 mM-Na₂EDTA, pH 7.4) from total HDL or their chromatographic fractions were mixed with 400 μl of LPDS (cholesterol concentration < 5 mg/dl). Two 50 μl samples were taken for cholesterol determination, and to the remaining solution 50 μl of heparin–Mn²⁺ reagent was added, and the mixture processed as above. Cholesterol concentration was determined in the supernatant, and the percentage of cholesterol precipitated was calculated from the differences between these values.

RESULTS AND DISCUSSION

Labelling of HDL with radioactive cholesteryl esters

The labelling of HDL particles with radioactive cholesteryl esters in the present work has been achieved by using two different approaches. One used [³H]cholesterol oleate dissolved in human LPDS [8]. The other consisted of the incubation of HDL with [¹⁴C]-labelled free cholesterol and its esterification by LCAT [12]. The conditions used in the present work were those previously described by others [8,12]. We have found that after incubation at 4 °C for 30 min with [³H]cholesterol oleate and human LPDS, both HDL₄ and HDL₃ incorporated more than 61% of the initial radioactivity, indicating highly efficient labelling. As shown in Fig. 1, which corresponds to HDL₂ (similar results were obtained with total HDL and HDL₂ results not shown), the specificity of the labelling was revealed by the coelution of radioactivity with protein and cholesterol in heparin–Sepharose column chromatography. The labelling, however, was not uniform, since specific radioactivity (d.p.m./μg of cholesterol) was markedly different in the three fractions (A, B and C respectively) obtained from either HDL₂ or HDL₃ (Table 1). These differences are maintained or even magnified if esterified cholesterol instead of total cholesterol is considered for specific-radioactivity calculation (results not shown), as the proportion of esterified versus total cholesterol decreases from A to C in the heparin–Sepharose-chromatography fractions [19]. The specific radioactivity attained in either total HDL₂ or total HDL₃ was much greater than that in their respective A or B fractions,
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Fig. 1. Elution profile of [3H]cholesterol oleate-labelled HDL₄ on heparin–Sepharose column chromatography

[3H]Cholesterol oleate-labelled HDL₄ (4.2 mg of cholesterol) dissolved in 2.5 ml of 0.05 M NaCl/5 mM Tris/HCl/25 mM MnCl₂, pH 7.4, was loaded on a chromatographic column (1.6 cm × 25 cm) containing heparin–Sepharose CL6-B. After equilibration at 4 °C, the column was eluted first with sample buffer (solution A), then with 0.095 M NaCl/5 mM Tris/HCl, pH 7.4 (solution B), and then with 0.29 M NaCl/5 mM Tris/HCl, pH 7.4 (solution C), at a flow rate of 24 ml/h. Fractions (1 ml) were collected, and $A_{280}$ (*) and total cholesterol (µg/ml) (△) were measured.

Table 1. Characteristics of radioactively labelled HDL and their subfractions isolated by heparin–Sepharose chromatography

Total HDL, HDL₂, and HDL₃ were labelled in vitro with either [3H]cholesterol oleate or [14C]cholesterol as indicated in the Materials and methods section. The lipoproteins were then subjected to heparin–Sepharose column chromatography, and three fractions (A, B, and C) were collected from each HDL preparation as described in the Materials and methods section.

<table>
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<tr>
<th>HDL/fraction</th>
<th>Protein/cholesterol mass ratio (mg/mg)</th>
<th>[3H] or [14C] sp. radioactivity (d.p.m./µg of cholesterol)</th>
<th>[3H] or [14C] radioactivity in esterified cholesterol (% of total)</th>
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<tr>
<td>[3H]Cholesterol oleate-labelled HDL</td>
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<td>Incubation conditions: 30 min at 4 °C</td>
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<td>Total HDL₄</td>
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<tr>
<td>HDL₂ A</td>
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<tr>
<td>HDL₂ B</td>
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<td>693</td>
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<tr>
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<tr>
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<tr>
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<td>Incubation conditions: 18 h at 37 °C</td>
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<tr>
<td>Total HDL</td>
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<tr>
<td>HDL C</td>
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<td>546</td>
<td>11.0</td>
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</table>

although these fractions together constitute more than 98% of the total cholesterol in any HDL₄ (Fig. 1) or HDL₃ (results not shown) preparation. The relatively high specific radioactivity of total HDL₄ or HDL₃ must then be due to the presence of a minor but intensely labelled fraction (fraction C). Our calculations show that, whereas fraction C contributes less than 2% of the cholesterol in total HDL₄ or HDL₃, it contains more than 47% of the radioactivity.

Highly efficient labelling with [3H]cholesterol oleate was also observed in subfractions from total HDL (Table 1). The three HDL subfractions obtained by heparin–Sepharose column chromatography showed different specific radioactivities, with that corresponding to fraction C being much higher than that of either fractions A or B (Table 1). The protein/cholesterol mass ratio of total HDL was similar to that found in fraction B, whereas the specific radioactivity was approx. 3 times greater,
and this finding was also observed with total HDL₂ and HDL₃. The cause of the distinct [³H]cholesterol olate incorporation into the HDL particles is still unknown.

To characterize the fractions better, unlabelled HDL₂ and HDL₃ were separately chromatographed on heparin–Sepharose columns as above and the fractions were processed for gel isoelectrofocusing [17]. As shown in Fig. 2, the major apoprotein in any fraction A and B from either HDL₂ or HDL₃ was apo A-I (A-Iₐ and A-Iₛ isomers). In HDL₂ fraction C, apo E and apo A-I were present in similar equivalent amounts. Apo A-II content relative to apo A-I, as determined by the ratio A-II/A-I + A-Iₛ from the densitometric scan (results not shown), in any fraction A was much higher than in any fraction B. In HDL₃ fraction C, apo A-II was undetectable by this technique. In fractions B, A-Iₛ and A-Iₐ isomers and traces of apo E were also detected. These results are in accordance with others that show the increase in the relative apo E content [4,5,19] and the decrease in apo A-II [19] of heparin–Sepharose fractions from A to C. These fractions also show some other differences between them, such as the protein/cholesterol mass ratio, which decreases from A to C (Table 1). So both the apoprotein/phospholipid mass ratio as well as the presence of apo A-II or apo E in the lipoprotein surface may contribute to the different labelling efficiency in HDL subfractions.

This inhomogeneity in labelling in vitro with [³H]cholesterol olate may have important consequences for the interpretation of the results obtained with this kind of preparation.

To find whether the distribution of radioactivity among the various HDL fractions was dependent on the time and the temperature of the incubation, total HDLs were mixed with [³H]cholesterol olate-containing LPDS as described above and incubated for 17 h at either 4 or 37°C and processed for heparin–Sepharose chromatography. Incubation for 17 h at 4°C produced fractions with very different [³H] specific radioactivities (555 d.p.m./µg of cholesterol in fraction A, 2337 d.p.m./µg in fraction B and 8631 d.p.m./µg in fraction C), as occurred when incubation was for 30 min (Table 1). However, when incubation was performed at 37°C for 17 h, [³H] specific radioactivity reached similar values among the fractions, which indicates that the tracer was homogeneously distributed among the lipoprotein particles, probably because the CETP-mediated reaction reached equilibrium under these conditions.

In the search for an alternative method for the isotopic labelling of HDL subfractions, HDL were incubated with free [¹⁴C] cholesterol at 37°C to allow the esterification reaction by plasma LCAT to take place [12]. We observed that the HDL particles were specifically labelled, inasmuch as [¹⁴C] radioactivity co-eluted with protein and cholesterol in heparin–Sepharose column chromatography (results not shown). Specific radioactivity of total HDL was similar to that of the major fraction (fraction B), and only minor differences in these parameters were observed between the three chromatographic fractions (Table 1). This result suggests that under our conditions the efficiency of free [¹⁴C] cholesterol incorporation into lipoprotein was similar in any HDL particle, which accords with previous results obtained by others [20]. However, whereas almost 35% of the [¹⁴C] cholesterol in fraction A was in the esterified form, this value was 28% in fraction B and only 11% in fraction C (Table 1). The last-mentioned results suggest that the LCAT esterification reaction took place at different rates depending on the composition of the lipoprotein particle, and was faster in HDL fraction A than in fraction C. This interpretation agrees with the observation by Marcel et al. [5] showing that the initial cholesterol esterification rate and Vmax shown in the HDL fraction not retained by heparin–Sepharose were always higher than those for the retained fraction. We did not investigate optimization of the labelling procedure, and it is not known whether, under other conditions or after prolonging the incubation time, the radioactive cholesterol esters formed by the LCAT action would equilibrate among the different HDL subfractions. We cannot, however, rule out the possibility that the different esterified [³H] cholesterol specific radioactivities among the HDL subfractions were the result of a preferential transfer of this lipid to fraction A from the other fractions by the action of the CETP present in human plasma. In any case, our results demonstrate that the incorporation of 'exogenous' labelled cholesterol esters, either directly by means of the neutral lipid transfer protein or else by LCAT action, is not readily related to the cholesterol content of the HDL particle, and this is responsible for the variations observed in the specific radioactivity achieved by the different HDL subfractions. These findings therefore oblige us to verify the homogeneity of the labelled HDL preparations in vitro before using them for metabolic studies.

Precipitation of [³H]cholesterol olate-labelled HDL subfractions by heparin–Mn²⁺

The separation of HDL from VLDL or LDL is an obvious requisite in most of the studies that are designed to analyse the CETP-mediated transfer of cholesterol esters between these lipoproteins. This separation is readily achieved by precipitation of VLDL and LDL with polyaniions and metals (see [21]), and several authors have already utilized this approach in this kind of study [8,10,12,22–24]. On the basis of the heterogeneous labelling of HDL subfractions observed under certain conditions, we considered the extent to which the HDL fractions, which had been prelabelled in vitro with [³H] cholesterol olate, were precipitated with the heparin–Mn²⁺ reagent [18] to be of interest. For this, total [³H]HDL₂, total [³H]HDL₃ and their respective heparin–Sepharose subfractions (A, B and C) were separately mixed with human LPDS, incubated for a certain period of time in the absence or presence of VLDL (as acceptor of the [³H]cholesterol esters), and then the lipoproteins were precipitated.
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Fig. 3. Precipitation of [3H]-cholesteryl oleate-labelled HDL2 and HDL3, and their subfractions by heparin-Mn2+

Portions (100 μl; 1.6 μg of esterified cholesterol) of 3H-labelled HDL2 or their subfractions isolated by heparin-Sepharose chromatography were mixed with 100 μl of 0.15 mM-NaCl/1 mM-Na2EDTA, pH 7.4, and 100 μl of human LPDS. The tubes were incubated for 0 or 2 h at 37 °C, and the lipoproteins were precipitated by addition of 0.7 ml of human serum and 0.1 ml of heparin (14.5 g/l)/1 mM-MnCl2. After mixing and centrifuging, the 3H radioactivity in the pellets was counted. Values correspond to the mean of two independent experiments which gave similar results. Dotted line ( ○ ○ ○ ○), total HDL2 or HDL3; thick line (△△△△), A fractions; dashed line (■■■■), B fractions; continuous thin line, (●●●●), C fractions.

by the addition of heparin-Mn2+ (final concns. 1.3 g/l and 91 mM respectively). At zero time of incubation, the initial radioactivity present in total HDL2 precipitated in a proportion greater than 76 %, whereas this precipitation was 9 %, 51 % and 91 % in HDL2 fractions A, B and C respectively (Fig. 3a). Similar results were observed with total HDL3 and its subfractions (Fig. 3b).

The high proportion of radioactivity precipitated from either total [3H]-HDL2 or HDL3, documented herein contrasts with the results obtained by others, who have observed that only a minor proportion of the radioactivity present in HDL was precipitated by heparin-Mn2+ [8,25]. Since these [3H]-labelled HDL preparations were not homogeneously labelled, this controversy could be partially due to the high precipitation of fraction C, which was the fraction showing the highest 3H specific radioactivity. To study this, we repeated the experiments but using homogeneously labelled HDL (those incubated at 37 °C for 17 h; Table 1). Total HDL and each of the subfractions were studied separately, and we found that the proportion of radioactivity precipitated from these fractions (16.6 % in fraction A, 42.5 % in fraction B and 82.0 % in fraction C) did not differ significantly from the values observed previously (Fig. 3). However, radioactivity precipitation from these homogeneously labelled total HDL preparations was much lower than from the inhomogeneously labelled ones (33 % versus 76 %), and this precipitation value corresponded to the average of the precipitation values of the fractions. Therefore, total HDL reflects the average of the subspecies that it contains only when they are homogeneously labelled.

Additional evidence was obtained by chemically determining the mass of cholesterol precipitated with the individual fractions and total HDL. For these experiments the mass of HDL-cholesterol in the tube was increased, and LPDS was used instead of whole serum. The results are shown in Fig. 4. First of all, it appeared that the percentage of cholesterol precipitated from both total HDL or subfractions was not detectably affected by the HDL concentration in the tube within the range studied. Fraction A was precipitated to a minimal extent (5–11 %), whereas fraction B was precipitated 32–38 % and fraction C was almost completely precipitated. The precipitation value with total HDL was 24–28 %, which was between those of fractions A and B, and therefore total HDL reflected the behaviour of the major subfractions when precipitation was evaluated by chemically measuring cholesterol.

All these results suggest that the high precipitation rate of both total [3H]-HDL2 and HDL3 (Fig. 3) was mainly due to the precipitation of fraction C. In other words, when labelled inhomogeneously, total HDL2 or HDL3 reflected the behaviour of their fraction with the highest specific radioactivity, even though it was in a minority, and this obliges us to recommend caution when interpreting the results obtained with total HDL labelled in vitro if homogeneous labelling is not confirmed.

The relatively large proportion (24–28 %) of HDL-cholesterol precipitated by the heparin-Mn2+ reagent, as determined chemically, was unexpected, but highly reproducible under our conditions, and was also observed with other precipitant reagents (dextran sulphate 50 000–Mg2+ and phosphotungstic acid-Mg2+; results not shown). For obvious reasons we used ultracentrifugally isolated HDL, which could be altered in some undetermined way, and the medium in which they were precipitated was not identical with plasma or serum. In this respect, it is known that the conditions for use of this kind of precipitant reagents have been highly adjusted to precipitate VLDL and LDL (but not HDL) in whole serum or plasma [21], and a minor change in the final concentrations of reagents or pH may lead to the incomplete precipitation of VLDL or LDL, or even to the precipitation of a significant amount of HDL [20,26,27]. Therefore our observations do not necessarily reflect the behaviour of intact HDL in serum under the action of the precipitant reagents. However, our results are in accordance with those of Puchois.
et al. [28], who demonstrated that both heparin–Mn²⁺ and dextran sulphate–Mg²⁺ reagents precipitated as much as 20% of total plasma concentrations of apo A-I and apo A-II in some normolipidaemic plasma samples, indicating that some HDLs were precipitated by these reagents.

One of the most relevant observations in the present work is the different precipitation rate of the three heparin–Sepharose subfractions, fraction A showing the lowest rate and fraction C the highest. Interestingly, the precipitating effect of heparin in these subfractions parallels their affinity for the heparin–Sepharose gel, suggesting a role for apo E in this effect. Actually, the interaction in vitro of apo E with heparin and the precipitation of apo E-rich HDL with this glycosaminoglycan, which have already been shown by others [29], may explain the high precipitation rate of HDL₃ fraction C. In addition to apo E, the different content of apo A-II as compared with apo A-I (higher in fraction A than in fraction B) may also be responsible for the different behaviour of these fractions, since apo A-II has been observed to be less effective than apo A-I in the formation of polyanion complexes [30]. Independently of the responsible mechanisms, our results demonstrate that the different HDL subpopulations behave differently under the precipitative action of heparin–Mn²⁺, which also accords with findings by Demacker et al. [27], who observed, with ¹²⁵I-labelled lipoproteins, that HDL₉₀ (d = 1.063–1.100) was more readily precipitable with dextran sulphate–Mg²⁺ than was HDL₃₀ (d = 1.100–1.125).

As observed in Fig. 3, the precipitation rate of any fraction B and C decreased after incubation with LPDS for 2 h at 37 °C. This was also observed with total HDL₂ and HDL₃, but not with fraction A when incubated separately. To determine if this phenomenon depends on incubation temperature, the HDL₂ subfractions were separately mixed with human LPDS and incubated for 0, 0.5 or 2 h at 4, 25 or 37 °C. It was observed that incubation at 37 °C, but not at 4 °C, produced a decrease in the precipitation rate of any fraction B and C, whereas fraction A was unaffected (Fig. 5). This phenomenon could be due either to a hypothetical change in the conformation of the lipoproteins, which made them less precipitable by heparin–Mn²⁺, or to the loss of [³H]cholesterol esters from those lipoproteins. To characterize this phenomenon further, experiments were conducted to check if those changes were attributable to the cholesterol ester transfer activity present in plasma. For this, HDL₂ subfractions were incubated in the presence of rat LPDS instead of human LPDS, and it was found that incubation for up to 2 h with rat LPDS did not affect the precipitation pattern of these lipoproteins (results not shown). Therefore, the changes observed with human LPDS (Fig. 5) may be ascribed to the transfer activity which it contains [31]. Although these incubations were performed in the absence of VLDL or LDL, which are the physiological acceptors of the cholesterol esters from HDL, the LPDS obtained by ultracentrifugation may have contained some free apoproteins as well as very-high-density lipoproteins [32,33], which could have acted in vitro as lipid acceptors and then have contributed to the decrease in the radioactivity-precipitation rate from HDL₂ fractions B and C observed after incubation at 37 °C in the presence of CETP-containing serum.
Transfer of [3H]cholesterol olate from prelabelled HDL fractions to VLDL

In order to study the transfer of [3H]cholesterol olate to VLDL, prelabelled HDL₂ and HDL₃, as well as their subfractions obtained by heparin-Sepharose chromatography, were incubated for 2 h with various concentrations of VLDL as the acceptor, in the presence of human or rat LPDS. Results corresponding to the incubation of 1.6 µg of HDL-esterified cholesterol in the presence of human LPDS at 37 °C are shown in Fig. 6. With any of the HDL subfractions studied, the addition of 30 µg of VLDL triacylglycerols produced an increase in the amount of 3H radioactivity that was precipitated by heparin–Mn²⁺. This increase took place at 37 °C, but not at either 4 °C or 25 °C, as observed in the HDL subfractions (Fig. 7). When rat LPDS was substituted for human LPDS, none of the effects mentioned were observed, although VLDL-triacylglycerol concentration was increased up to 30 µg/tube and temperature was 37 °C (results not shown). These results strongly suggest that the increase in 3H radioactivity precipitation observed in Fig. 6 was due to the transfer of some of the [3H]cholesterol olate to VLDL mediated by the CETP present in human LPDS. Besides demonstrating that this transfer process is dependent on the temperature, our observations with human compared with rat LPDS completely agree with previous ones by others [31], and prove our experimental approach valid for the analysis of the cholesteryl ester transfer activity.

In order to compare the capacity of the different HDL subfractions (A, B and C from HDL₂ and from HDL₃) as donors of cholesteryl esters in the CETP-mediated reaction, each prelabelled lipoprotein subfraction was incubated at different HDL-
cholesterol concentrations with VLDL for 2 h at 37 °C in the presence of a fixed volume of human LPDS, and the reaction was stopped by lipoprotein precipitation as described above. The transfer of cholesteryl oleate to VLDL (in μg of esterified cholesterol/2 h per tube) was calculated by subtracting the precipitation value obtained in the absence of VLDL from that in its presence, and making the appropriate correction for the concentration and 3H specific radioactivity of HDL. Values correspond to the mean of two independent experiments which gave similar results.

As observed in Figs. 8 and 9, the transfer of cholesteryl oleate to VLDL from any of the HDL subfractions studied increased as a function of both HDL cholesterol and VLDL triacylglycerol concentrations in the media. Except for HDL2 fraction C, curves corresponding to the other HDL2 (Fig. 8) and HDL3 (Fig. 9) subfractions could be satisfactorily adjusted to each hyperbola, the maximum of which depended on the amount of VLDL present in the media. This indicates that the transfer of cholesteryl oleate from any HDL subfraction in the medium to VLDL was stimulated by donor concentration and limited by the acceptor availability, confirming previous observations by others [25,34–36]. The apparently distinct behaviour of HDL3 fraction C (rectilinear rather than hyperbolic curve) was probably the result of the different donor/acceptor mass ratio used in this case (10 times less than for fractions A or B), owing to the low availability of this fraction. The maximum amount of esterified cholesterol transferred to different amounts of VLDL during 2 h was estimated as the asymptote of each hyperbola and, for HDL fraction A, was 1.50 μg of esterified cholesterol with 6.9 μg of VLDL triacylglycerol, 6.22 μg with 30 μg of VLDL triacylglycerol and 11.78 μg with 60 μg of VLDL triacylglycerol. When expressed in μg of HDL-esterified cholesteryl/μg of VLDL-triacylglycerol, these values were 0.22, 0.21 and 0.20 respectively, indicating that the maximum transfer of cholesteryl esters from HDL3 fraction A to VLDL was determined by the acceptor rather than by the donor. This was also true for the other HDL subfractions.

The mean maximum transfer of esterified cholesterol to VLDL (in μg/μg of VLDL triacylglycerol) was estimated to be 0.16, 0.13 and 0.12 with HDL2 fraction A, HDL2 fraction B and HDL3 fraction B respectively. These results thus show the similarities between the A fractions on one hand, and the B fractions on the other, and indicate that fraction A, whether from HDL3 or from HDL4, is a better substrate in vitro for CETP action than is fraction B. To study this phenomenon more carefully, transfer results were represented against the VLDL concentration in media. As shown in Fig. 10, which corresponds to the results obtained with the intermediate concentration of HDL studied (6.8 μg of esterified cholesterol/tube), at every VLDL concentration the transfer of cholesteryl esters from HDL3 fraction A was higher than from HDL3 fraction B, and similar differences were observed with the HDL4 subfractions. Although the experimental conditions did not reflect initial velocities, and saturation was not reached in our studies, an estimate of the apparent Vmax could be derived mathematically. These values (in μg of esterified cholesterol/2 h) were 5.46 with HDL2 fraction A, 4.60 with HDL2 fraction B, 5.35 with HDL3 fraction A and 4.65 with HDL3 fraction B. These results again demonstrate the differences between fractions A and B, and the similarities between the analogous fractions from HDL2 and HDL3. The apparent Km values were practically the same in every case (40.4, 41.1, 42.0 and 48.0 μg of VLDL triacylglycerol/tube for each respective subfraction), which indicates that differences in the cholesteryl ester transfer rate observed between the HDL subfractions were not influenced by the acceptor, the VLDL.
in vitro may show distinct specific activities under certain conditions and that they behave differently both in biochemical reactions and in the separation methods (including the commonly used polyanion–metal precipitation) obliges us to be very cautious when results obtained with total HDL are interpreted, and to seek labelling conditions that produce homogeneously labelled lipoprotein particles.

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