

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

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1. Human total HDL (high-density lipoprotein), HDL₂ and HDL₃ were labelled *in vitro* by incubation with lipoprotein-deficient serum (LPDS) which contained either [³H]cholesteryl oleate or [¹⁴C]cholesterol under different conditions. The lipoproteins were then subfractionated by heparin–Sepharose column chromatography, and three subfractions (A, B and C) were successively eluted from each preparation of HDL, HDL₂ and HDL₃. When the labelling was done at 37 °C for 17 h, the subfractions were homogeneously labelled with [³H]cholesteryl oleate. However, when it was performed for only 30 min at 4 °C, the subfractions showed marked differences in the ³H specific radioactivity, which was much higher in the C fractions than in the others. 2. ³H-labelled HDL₂ and HDL₃ subfractions behaved differently under the precipitant action of heparin–Mn²⁺; fraction C (the richest in apolipoprotein E) produced the largest amount of radioactive and chemical precipitate. More ³H radioactivity, but not the cholesterol, was precipitated from HDL₂ or HDL₃ by the reagent, demonstrating that ³H-labelled HDL₂ and HDL₃ behave like their fraction C, which becomes labelled to the highest specific radioactivity despite having the smallest mass. 3. The incubation of ³H-labelled HDL subfractions with human LPDS and very-low-density lipoprotein (VLDL) at 37 °C increased the quantity of ³H radioactivity that was precipitated, in proportion to the amount of VLDL present in the media. These changes were attributable to the action of cholesterol ester transfer protein, since they did not occur at 4 °C or when human LPDS was replaced with rat LPDS. 4. Kinetics of the transfer of HDL [³H]cholesteryl oleate to VLDL showed a greater apparent V_{max} for fractions A than for fractions B from either HDL₂ or HDL₃, whereas the apparent K_m values were very similar, which suggest that this transfer process is influenced by the apoprotein composition of the donor lipoprotein.

INTRODUCTION

Both human HDL₂ and HDL₃ are heterogeneous and poly-disperse 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 HDL₂ and HDL₃ 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 obviously demands 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, HDL₂ or HDL₃ 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 ($d < 1.006$), HDL ($d = 1.063–1.21$), HDL₂ ($d = 1.063–1.125$), 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 normolipidaemic 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 μ Ci of [1,2,6,7-³H]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, lecithin: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]cholesteryl oleate-labelled HDL₂ ($d = 1.125$), HDL₃ 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 M-NaCl/1 mM-Na₂EDTA, pH 7.4.

Labelling with [¹⁴C]cholesterol

Approx. 4 μ Ci 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 M-NaCl/1 mM-Na₂EDTA, pH 7.4. Radioactivity recovery in the HDL fraction was over 76%.

HDL fractionation by heparin–Sephacryl affinity chromatography

When further subfractionation by heparin–Sephacryl column chromatography was undertaken, the radioactively labelled HDL preparations were dialysed against 0.05 M-NaCl/5 mM-Tris/HCl, pH 7.4, then supplemented with solid MnCl₂ to give a final concentration of 25 mM and loaded into chromatography columns containing heparin–Sephacryl 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 M-NaCl/25 mM-MnCl₂ (solution A), 0.095 M-NaCl (solution B) or 0.29 M-NaCl (solution C). Fractions were collected and processed for measurement of A_{280} , 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 M-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/diethyl ether/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 μ m-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 M-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]cholesteryl oleate to VLDL, 100 μ l of prelabelled total HDL₂, total HDL₃ or their heparin–Sephacryl-chromatography fractions, containing 1.6, 6.8 or 15.4 μ g of esterified cholesterol respectively, were mixed with 100 μ l of VLDL suspension containing 0–60 μ g of triacylglycerol in 0.15 M-NaCl/1 mM-Na₂EDTA, pH 7.4, and 100 μ l of human or rat LPDS. The tubes were incubated at 4, 25 or 37 °C for 0

30 or 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 M) [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]cholesteryl oleate from HDL to VLDL (expressed as μ g 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]cholesteryl oleate specific radioactivity in HDL.

To determine the mass of cholesterol precipitated by the heparin–Mn²⁺ reagent, different amounts of HDL-cholesterol (in 200 μ l of 0.15 M-NaCl/1 mM-Na₂EDTA, pH 7.4) from total HDL or their chromatographic fractions were mixed with 400 μ l of LPDS (cholesterol concentration < 5 mg/dl). Two 50 μ l samples were taken for cholesterol determination, and to the remaining solution 50 μ l 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]cholesteryl 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]cholesteryl oleate and human LPDS, both HDL₂ 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 HDL₃; results not shown), the specificity of the labelling was revealed by the co-elution of radioactivity with protein and cholesterol in heparin–Sephacryl column chromatography. The labelling, however, was not uniform, since specific radioactivity (d.p.m./ μ g 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–Sephacryl-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,

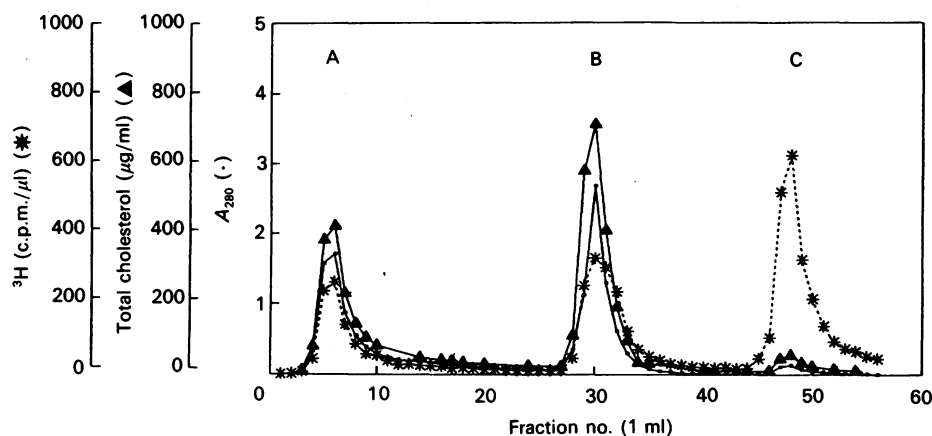


Fig. 1. Elution profile of [^3H]cholesteryl oleate-labelled HDL₂ on heparin-Sepharose column chromatography

[^3H]cholesteryl oleate-labelled HDL₂ (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 \times 25 cm) containing heparin-Sepharose CL6-B. After equilibration at 4 $^{\circ}\text{C}$, 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} (\cdot), ^3H radioactivity (d.p.m./ μl) ($*$) and total cholesterol ($\mu\text{g}/\text{ml}$) (\blacktriangle) 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]cholesteryl oleate or [^{14}C]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.

| HDL/fraction | Protein/cholesterol mass ratio (mg/mg) | ^3H or ^{14}C sp. radioactivity (d.p.m./ μg of cholesterol) | ^3H or ^{14}C radioactivity in esterified cholesterol (% of total) |
|---|--|--|--|
| [^3H]cholesteryl oleate-labelled HDL | | | |
| Incubation conditions: 30 min at 4 $^{\circ}\text{C}$ | | | |
| Total HDL ₂ | 2.94 | 2392 | — |
| HDL ₂ A | 3.81 | 618 | — |
| HDL ₂ B | 2.72 | 693 | — |
| HDL ₂ C | 1.64 | 10497 | — |
| Total HDL ₃ | 4.66 | 4431 | — |
| HDL ₃ A | 4.67 | 473 | — |
| HDL ₃ B | 4.40 | 1244 | — |
| HDL ₃ C | 2.34 | 41070 | — |
| Total HDL | 3.63 | 3127 | 97.1 |
| HDL A | 4.39 | 885 | 96.6 |
| HDL B | 3.41 | 1262 | 97.2 |
| HDL C | 1.78 | 5986 | 94.3 |
| Incubation conditions: 17 h at 37 $^{\circ}\text{C}$ | | | |
| Total HDL | 3.81 | 2328 | — |
| HDL A | 4.13 | 2665 | — |
| HDL B | 3.62 | 2264 | — |
| HDL C | 1.28 | 2214 | — |
| [^{14}C]cholesterol-labelled HDL | | | |
| Incubation conditions: 18 h at 37 $^{\circ}\text{C}$ | | | |
| Total HDL | 3.63 | 568 | 29.3 |
| HDL A | 4.48 | 473 | 34.7 |
| HDL B | 3.30 | 577 | 27.8 |
| HDL C | 1.56 | 546 | 11.0 |

although these fractions together constitute more than 98 % of the total cholesterol in any HDL₂ (Fig. 1) or HDL₃ (results not shown) preparation. The relatively high specific radioactivity of total HDL₂ 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 HDL₂ or HDL₃, it contains more than 47 % of the radioactivity.

Highly efficient labelling with [^3H]cholesteryl 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,

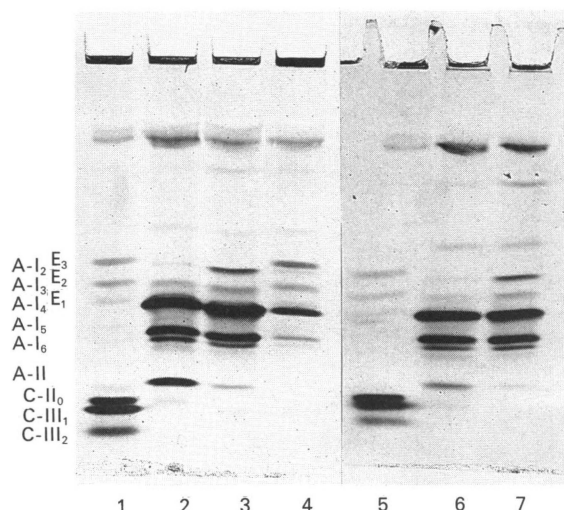


Fig. 2. Gel isoelectrofocusing of delipidated HDL₂ and HDL₃ subfractions

HDL₂ and HDL₃ subfractions isolated by heparin-Sepharose chromatography were delipidated with ethanol/acetone (1:1, v/v), resuspended in 8 M-urea/10 mM-dithiothreitol/10 mM-Tris/HCl, pH 8.6, and subjected to isoelectrofocusing in the pH range 4–6.5 in 5.5% polyacrylamide slab gels. HDL₃ fraction C could not be run because of low availability. VLDLs were run for identification of apo E and apo C species. Lanes: 1, VLDL; 2, HDL₃ fraction A; 3, HDL₂ fraction B; 4, HDL₂ fraction C; lane 5, VLDL; lane 6, HDL₃ fraction A; lane 7, HDL₃ fraction B.

and this finding was also observed with total HDL₂ and HDL₃. The cause of the distinct [³H]cholesteryl oleate 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-I₄ and A-I₅ isoforms). 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-I₄ + A-I₅ from the densitometric scan (results not shown), in any fraction A was much higher than in any fraction B. In HDL₂ fraction C, apo A-II was undetectable by this technique. In fractions B, A-I₂ and A-I₃ isoforms 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]cholesteryl oleate 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]cholesteryl oleate-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 V_{max} 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 cholesteryl 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 cholesteryl 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]cholesteryl oleate-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 cholesteryl esters between these lipoproteins. This separation is readily achieved by precipitation of VLDL and LDL with polyanions 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]cholesteryl oleate, 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]cholesteryl esters), and then the lipoproteins were precipitated

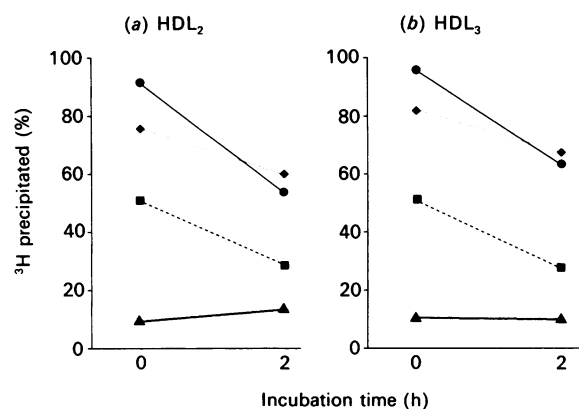


Fig. 3. Precipitation of [³H]-cholesteryl oleate-labelled HDL₂ and HDL₃, and their subfractions by heparin-Mn²⁺

Portions (100 μ l; 1.6 μ g of esterified cholesterol) of ³H-labelled HDL₂, HDL₃ or their subfractions isolated by heparin-Sephacryl chromatography were mixed with 100 μ l of 0.15 M-NaCl/1 mM-Na₂EDTA, pH 7.4, and 100 μ l 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 M-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

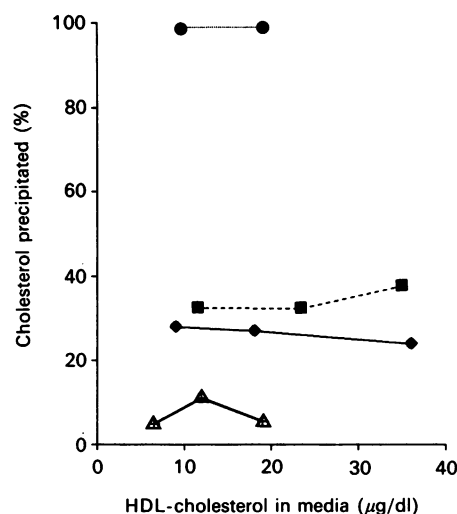


Fig. 4. Effect of HDL-cholesterol concentration in media on the heparin-Mn²⁺ precipitation of cholesterol from total HDL and each of their subfractions

Samples (200 μ l) of any unlabelled HDL and each of their subfractions isolated by heparin-Sephacryl chromatography were mixed with 400 μ l of LPDS, and the cholesterol concentration was measured in two 50 μ l portions. To the remaining 500 μ l, 50 μ l of heparin (14.5 g/l)/1 M-MnCl₂ was added. After mixing and centrifuging, the cholesterol concentration in the supernatant was determined. Precipitation was calculated by the difference between these cholesterol values, and was expressed as a percentage of the initial HDL-cholesterol concentration in the medium. Values correspond to the means of triplicates. Continuous thin line (◆—◆), total HDL; thick line (▲—▲), HDL fraction A; dashed line (■----■), HDL fraction B; dotted line (●····●), HDL fraction C.

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 50000-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

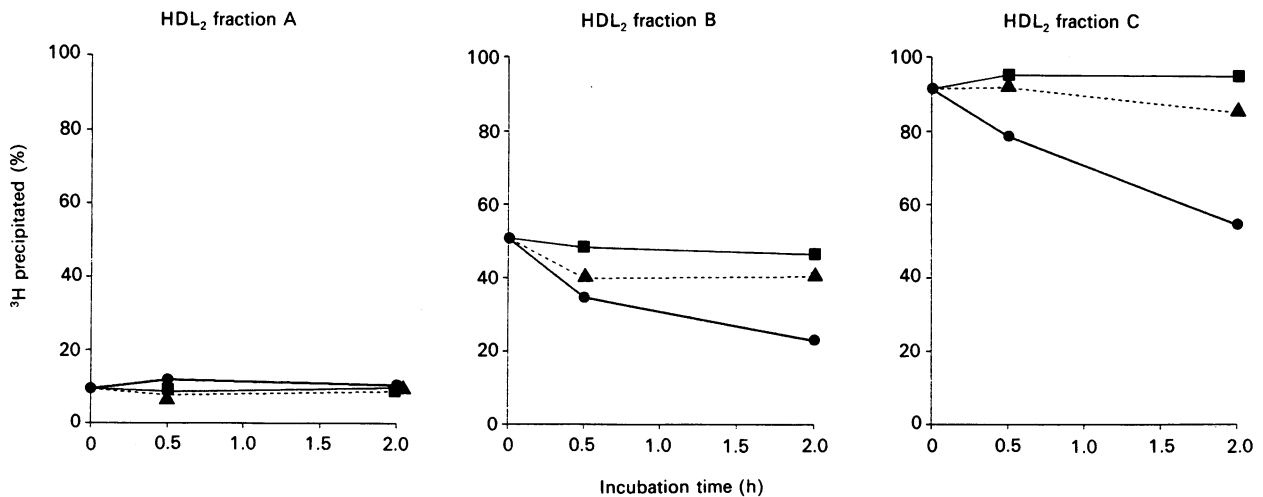


Fig. 5. Effect of temperature on heparin- Mn^{2+} precipitation of [3H]cholesteryl oleate-labelled HDL₂ subfractions incubated with human LPDS

Samples (100 μ l; 1.6 μ g of esterified cholesterol) of HDL₂ subfractions isolated by heparin-Sephacryl chromatography (A, B and C) were mixed with human LPDS as in Fig. 3 and incubated for 0, 0.5 and 2 h at 4, 25 or 37 °C. Values correspond to the mean of two independent experiments which gave similar results. Continuous thin line (■—■), 4 °C; dashed line (▲---▲), 25 °C; thick line (●—●), 37 °C.

et al. [28], who demonstrated that both heparin- Mn^{2+} and dextran sulphate- Mg^{2+} 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-Sephacryl 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-Sephacryl 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 HDL₂ 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- Mn^{2+} , which also accords with findings by Demacker *et al.* [27], who observed, with ^{125}I -labelled lipoproteins, that HDL_{2b} ($d = 1.063$ – 1.100) was more readily precipitable with dextran sulphate- Mg^{2+} than was HDL_{2a} ($d = 1.100$ – 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- Mn^{2+} , or to the loss of [3H]cholesteryl esters from those lipoproteins. To characterize this phenomenon further, experiments were conducted to check if those changes were attributable to the cholesteryl ester transfer activity present in plasma. For this,

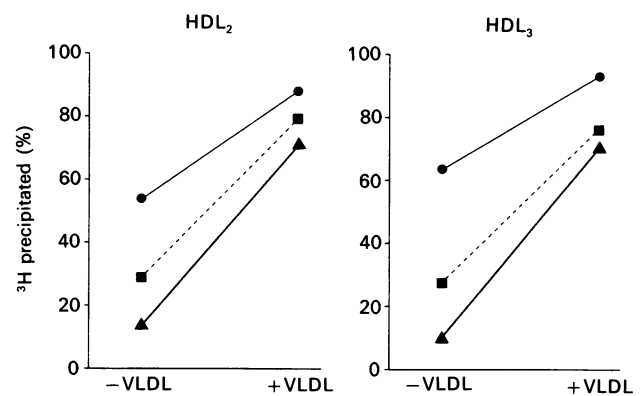


Fig. 6. Effect of VLDL on heparin- Mn^{2+} precipitation of [3H]cholesteryl oleate-labelled HDL subfractions incubated with human LPDS

Samples (100 μ l; 1.6 μ g of esterified cholesterol) of 3H -labelled HDL₂ or HDL₃ subfractions isolated by heparin-Sephacryl chromatography (A, B and C from each) were mixed with 100 μ l of human VLDL (30 μ g of triacylglycerol) in 0.15 M-NaCl/1 mM- Na_2EDTA , pH 7.4, and 100 μ l of human LPDS. The tubes were incubated for 2 h at 37 °C and processed as in Fig. 3. Values correspond to the mean of two independent experiments which gave similar results. Thick lines (▲—▲), A fractions; dashed lines (■---■), B fractions; continuous thin lines (●—●), C fractions.

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 cholesteryl 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]cholesteryl oleate from prelabelled HDL fractions to VLDL

In order to study the transfer of [³H]cholesteryl oleate to VLDL, prelabelled HDL₂ and HDL₃, as well as their subfractions obtained by heparin–Sephacryl 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]cholesteryl oleate 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-

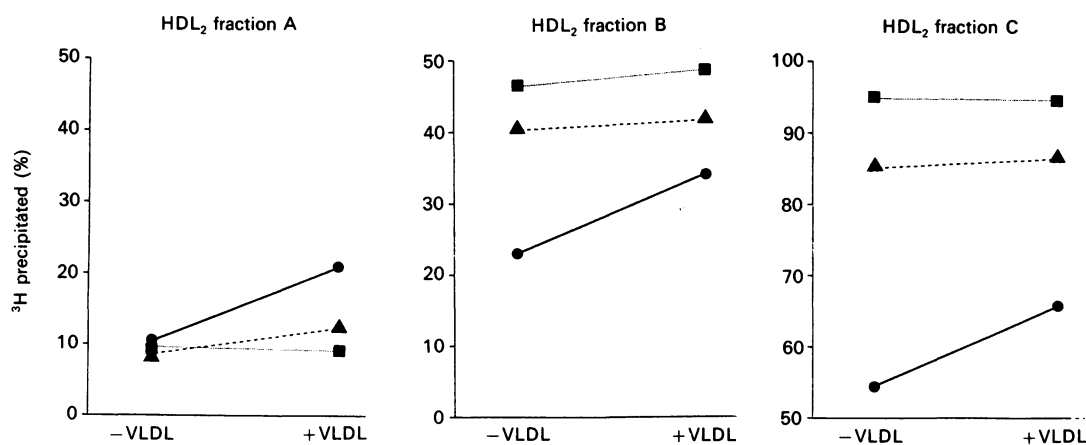


Fig. 7. Effect of temperature on heparin–Mn²⁺ precipitation of [³H]cholesteryl oleate-labelled HDL₂ subfractions incubated with human LPDS and VLDL.

Samples (100 μl; 1.6 μg of esterified cholesterol) of ³H-labelled HDL₂ subfractions (A, B and C) isolated by heparin–Sephacryl chromatography were mixed with VLDL and human LPDS as in Fig. 6, and incubated for 2 h at 4, 25 or 37 °C. The tubes were processed as in Fig. 3. Values correspond to the mean of two independent experiments which gave similar results. Continuous thin line (■—■), 4 °C; dashed line (▲----▲), 25 °C; thick line (●—●), 37 °C.

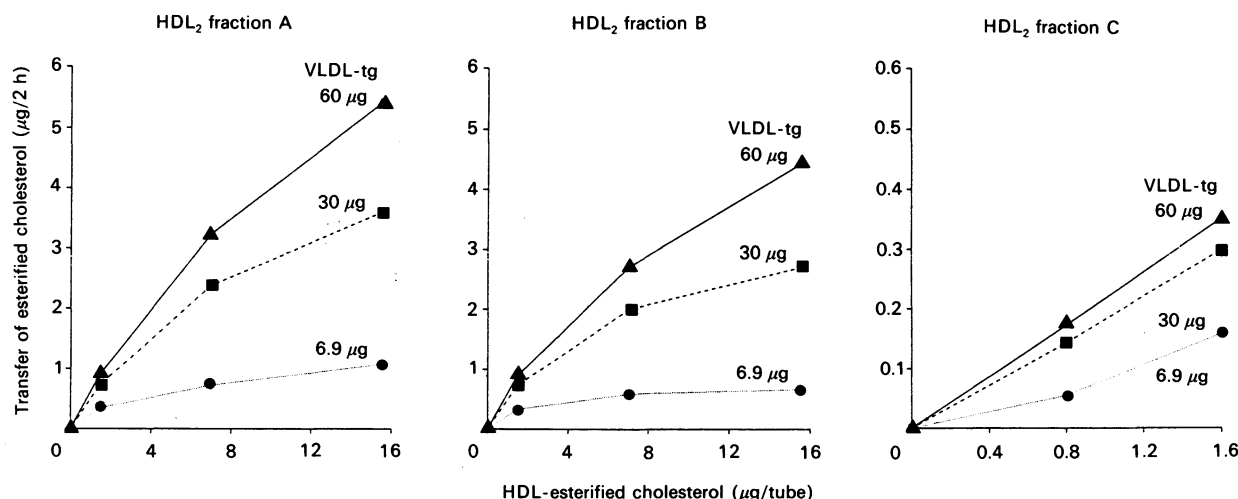


Fig. 8. Transfer of [³H]cholesteryl oleate from prelabelled HDL₂ subfractions to VLDL by effect of human LPDS

Samples (100 μl) of [³H]cholesteryl oleate-labelled HDL₂ subfractions containing 1.6, 6.8 or 15.4 μg of esterified cholesterol, were mixed with 0, 6.9, 30 or 60 μg of human VLDL triacylglycerol (VLDL-tg) in 100 μl of 0.15 M-NaCl/1 mM-Na₂EDTA, pH 7.4, and 100 μl of human LPDS. The tubes were incubated at 37 °C for 2 h and processed as in Fig. 3. The transfer of cholesteryl oleate to VLDL was calculated by subtracting the precipitation value 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.

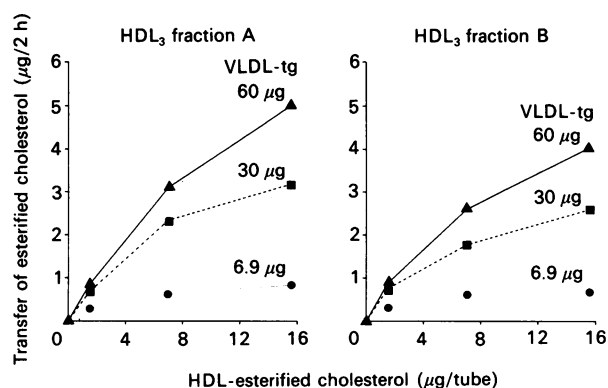


Fig. 9. Transfer of [^3H]cholesteryl oleate from prelabelled HDL₃ subfractions to VLDL

Samples (100 μl) of [^3H]cholesteryl oleate-labelled HDL₃ subfractions containing 1.6, 6.8 or 15.4 μg of esterified cholesterol were mixed with 0, 6.9, 30 or 60 μg of human VLDL triacylglycerol (VLDL-tg) in 100 μl of 0.15 M-NaCl/1 mM-Na₂EDTA, pH 7.4, and 100 μl of human LPDS. The tubes were incubated at 37 $^{\circ}\text{C}$ for 2 h and processed as in Fig. 3. The transfer of cholesteryl oleate to VLDL was calculated by subtracting the precipitation value 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.

cholesterol concentrations with VLDL for 2 h at 37 $^{\circ}\text{C}$ 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 the presence of VLDL, and making the appropriate correction for the concentration and ^3H specific radioactivity of the HDL subfraction used.

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 HDL₂ 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 cholesterol/ μg of VLDL-triacylglycerol, these values were 0.22, 0.21 and 0.20 respectively, indicating that the maximum transfer of cholesteryl esters from HDL₂ 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 $\mu\text{g}/\mu\text{g}$ of VLDL triacylglycerol) was estimated to be 0.16, 0.13 and 0.12 with HDL₃ fraction A, HDL₂ fraction B and HDL₃

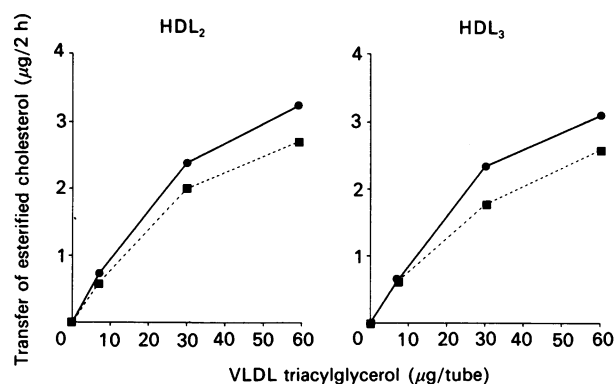


Fig. 10. Comparison of the transfer of [^3H]cholesteryl oleate from prelabelled HDL₂ and HDL₃ subfractions to VLDL

Samples (100 μl ; 6.8 μg of esterified cholesterol) of ^3H -labelled HDL₂ or HDL₃ subfractions isolated by heparin-Sepharose chromatography were mixed with VLDL and human LPDS. Incubation conditions and calculations were as indicated in Figs. 8 and 9. Values correspond to the mean of two independent experiments which gave similar results. Thick lines (●—●), A fractions; dashed lines (■---■), B fractions.

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 HDL₂ or from HDL₃, 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 HDL₂ fraction A was higher than from HDL₂ fraction B, and similar differences were observed with the HDL₃ subfractions. Although the experimental conditions did not reflect initial velocities, and saturation was not reached in our studies, an estimate of the apparent V_{max} 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 K_m 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.

Since cholesteryl ester mass was not determined in the present experiments, we are not certain whether or not the transfer of [^3H]cholesteryl oleate that we observed represents a net transfer of cholesteryl ester from HDL to VLDL. In spite of this, our results show that all HDL subfractions are substrates for CETP, and the kinetic parameters of the process are distinct for each HDL fraction. Fraction A showed an apparent V_{max} significantly higher than fraction B, from either HDL₂ or HDL₃ (Fig. 10). Since the most striking difference between these fractions is the apoprotein composition (apo A-I/apo A-II mass ratio and apo E content are lower in fraction A than in fraction B; the present work, and ref. [19]), a role for these apoproteins in the transfer reaction could be envisaged, but this obviously needs to be confirmed *in vivo*.

The heterogeneity of HDL has not only metabolic relevance but also important methodological implications, as observed herein. Our demonstration that the HDL subfractions labelled

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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