Dynamic properties of human high density lipoprotein apoproteins

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Abstract This study was designed to identify a method for the measurement of human high density lipoprotein subfraction (HDL_2 and HDL_3) metabolism. Apolipoproteins A-I, A-II, and C, the major HDL apoproteins, were radioiodinated and incorporated individually into HDL_2 and HDL_3 in vitro. Using a double label technique, the turnover of apoA-I in HDL_2 and HDL_3 was measured simultaneously in a normal male. The apoprotein exchanged rapidly between the two subfractions, evidenced by equilibration of their apoA-I specific activity. Radiolabeled apoA-II, incorporated into the subfractions, showed a similar exchange in vitro. Incubation of ^{131}I-labeled very low density lipoproteins (VLDL) with HDL or its subfractions resulted in transfer of C proteins from VLDL to the HDL moiety. The extent of transfer was dependent on the HDL subfraction present; 50% of the VLDL apoC was transferred to HDL_3, while the transfer to total HDL and HDL_2 was 69% and 78%, respectively. ApoC also exchanged between HDL_2 and HDL_3, again showing a preference for the former and suggesting a primary metabolic relationship between VLDL and HDL_2. Overall, the study indicates that apoA-I, apoA-II, and the C proteins exist in equilibrium between HDL_2 and HDL_3. This phenomenon precludes their use as probes for HDL subfraction metabolism in humans.

Supplementary key words HDL subfraction metabolism · rate zonal ultracentrifugation · apolipoprotein exchange · apolipoproteins A-I, A-II and C

Plasma high density lipoproteins are customarily divided into two density classes, HDL_2 and HDL_3, which differ in composition (1) and size (2), and appear to be two distinct lipoprotein populations on the basis of analytical (3) and rate zonal (1) ultracentrifugation. The functions of these two species are not yet understood but a number of reports indicate that each may be metabolized independently of the other. For example, premenopausal women have higher plasma HDL_2 levels than men of the same age (4) and, as a result, may derive protection from atherosclerosis (5, 6). Perturbations of plasma HDL subfraction concentrations can also be produced by various therapeutic measures. Nicotinic acid therapy induces an increase in the ratio of plasma HDL_2/HDL_3 (7) while estrogen treatment of normal subjects causes a selective rise in HDL_3 levels without influencing significantly plasma HDL_2 (8).

In order to pursue the metabolic factors responsible for these changes in HDL subfraction distribution, we have examined the constituent apoproteins of HDL to determine their value as probes for the investigation of HDL subfraction metabolism. Our findings are presented in this report.

EXPERIMENTAL METHODS

Preparation of HDL_2 and HDL_3

Rate zonal ultracentrifugation was employed to isolate two high density lipoprotein subclasses, HDL_2 and HDL_3. A nonlinear density gradient of NaBr (d 1.00–1.40 g/ml) in 350 μM disodium EDTA (pH 7.6) was established in a Beckman Ti-14 zonal rotor (Beckman Instruments, Palo Alto, CA) using a Beckman model 141 gradient pump. Fifteen-milliliter aliquots of fresh postabsorptive plasma were fractionated in this system at 41,000 rpm and 10°C for 21 hr. After ultracentrifugation, the rotor contents were monitored continuously at 280 nm and collected in 10-ml aliquots. The rate zonal procedure has been detailed elsewhere (1); a typical ultracentrifuge elution profile is shown in **Fig. 1**.

The fractions corresponding to HDL_2 and HDL_3

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins, d 1.063–1.210 g/ml; HDL_2, high density lipoproteins, d 1.063–1.125 g/ml; HDL_3, high density lipoproteins, d 1.125–1.210 g/ml; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; apoC, apolipoprotein C; SDS, sodium dodecyl sulfate.

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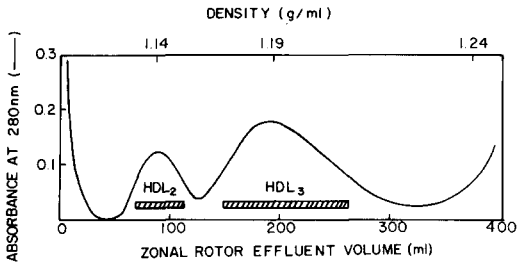

Fig. 1. Preparation of HDL_2 and HDL_3 by rate zonal ultracentrifugation. HDL_2 and HDL_3 were isolated from 15-ml aliquots of fresh postabsorptive plasma by centrifugation into a nonlinear NaBr gradient (d 1.00–1.40 g/ml). The peak fractions (hatched) were pooled.

were pooled separately and dialyzed exhaustively against 0.05 M barbital buffer, pH 8.6, prior to their labeling by the in vitro transfer procedure described below.

Purification and labeling of apolipoproteins A-I and A-II

HDL was isolated from normal fasting donors by ultracentrifugation in the density range 1.063–1.21 g/ml as described previously (9), and delipidated using diethyl ether–ethanol 3:1 (10). Apolipoproteins A-I and A-II were fractionated on G-150 Sephadex (9) using 0.1 M Tris-HCl, pH 8.6, containing 5.4 M urea and 0.1% disodium EDTA as elution buffer. The purity of the apoproteins was confirmed by amino acid analysis, acrylamide gel electrophoresis (11), and crossed immunoelectrophoresis (12). The total amino acid composition of each apoprotein was in agreement with that reported by others (13, 14). Preparations that failed to meet these criteria were rechromatographed. The apoproteins were dialyzed against 0.01 M Tris buffer, pH 7.0, containing 0.15 M NaCl and 0.01% disodium EDTA. Antibodies were raised in rabbits against the purified apoA-I (15); crossed immunoelectrophoresis of whole plasma showed that the antisera were monospecific.

The purified apoA-I and apoA-II were radioiodinated with ^{131}I and ^{125}I (Amersham-Searle, Arlington Heights, IL) by a modification (16) of the McFarlane technique (17) in which the reaction mixture contained 50 nmol of apoprotein, 100 nmol of ICl, and 2 mCi of $Na^{125}I$ or $Na^{131}I$ in 1.5 ml of 1.0 M glycine buffer, pH 10.0. Immediately after mixing, the iodinated protein was separated from unbound radioiodide by gel filtration through a 1.0 × 25 cm Sephadex G15 column. The eluting buffer contained 0.1 M Tris-HCl, pH 8.6, 0.15 M NaCl, and 0.01% disodium EDTA. Labeling efficiency was approximately 50%. The labeled apoprotein was dialyzed against 0.05 M barbital buffer (pH 8.6) until paper electrophoresis showed that less than 1% free radioiodide remained in the preparation.

Labeling of HDL_2 and HDL_3

Labeling with radioiodinated apoA-I and apoA-II. HDL_2 and HDL_3, prepared by rate zonal ultracentrifugation, were labeled by in vitro incubation with radioiodinated apoA-I or apoA-II. One nmol of labeled apoprotein was incubated with a 20-fold molar excess of HDL_2 or HDL_3 (estimated on the basis of protein (18), and assuming that each mol of HDL_2 or HDL_3 contains 125,000 g of protein) for 30 min at 25°C in 8.0 ml of 0.05 M barbital buffer, pH 8.6. The solution density was then raised to 1.225 g/ml by addition of solid KBr and unbound apoprotein separated from the lipoprotein by ultracentrifugation (20 hr at 55,000 rpm, 10°C) in a Beckman 65 anglehead rotor.

Aliquots of 1.0 ml were removed progressively from the top of the centrifuge tube and counted in a Packard Autogamma spectrometer (Packard Instrument Co., Inc., Downers Grove, IL). The top three fractions from each tube, containing the apoprotein-labeled HDL subfractions were pooled and dialyzed against 0.05 M barbital buffer, pH 8.6 prior to in vitro studies, or 0.01 M Tris, 0.15 M NaCl, 0.01% disodium EDTA before use in vivo.

Labeling with apoC. HDL or its subfractions were labeled with apoC by in vitro transfer from VLDL. VLDL from a type IV hyperlipoproteinemic subject was isolated and washed once using standard ultracentrifugation procedures (19) which freed it from other plasma proteins or lipoproteins. This was then radiolabeled with ^{131}I by a modification of the McFarlane technique (16). The radioactivity distribution was 31% in apoB, 45% in apoC (as estimated by the method of Kane (20)), and 24% in lipid. Radioactivity was not detectable in the small amounts of apoA-I and apoA-II present in VLDL. Incubation of the ^{131}I-VLDL (1.2 mg of protein) with HDL or its subfractions (2.5 mg of protein) at 37°C for 30 min in 8.0 ml of 0.05 M barbital buffer, pH 8.6, resulted in a transfer of radioactivity from VLDL to HDL. The lipoproteins were then separated by ultracentrifugation at a density of 1.020 g/ml in 10-ml capacity polycarbonate tubes (Beckman anglehead 65 rotor, 20 hr, 55,000 rpm, 10°C). No more than 10% of the radioactivity that transferred to HDL or its subfractions was extractable into ether–ethanol 3:1, and SDS polyacrylamide gel electrophoresis showed that the remainder was associated with apoC. That the transferred material was firmly bound to HDL was confirmed by rate zonal ultracentrifugation (see below).

In vivo transfer of apoA-I between HDL subfractions

This study was undertaken with the approval of the Human Research Committee of Baylor College of Medicine and The Methodist Hospital. A healthy normolipemic adult male subject (age 28 years, weight 75 kg) gave informed consent to the project. For 3 days prior to and throughout the study he received 900 mg of KI per day in divided doses to prevent thyroidal uptake of radioiodide. He took no other medication.

Twenty-five μCi of autologous ^{125}I-apoA-I/HDL$_2$ (0.5 mg of protein) were mixed with 25 μCi of ^{131}I-apoA-I/HDL$_3$ (1.0 mg of protein). The mixture was sterilized by filtration (0.22 μm Millipore filters, Millipore Corp., Bedford, MA) and immediately administered to the volunteer by the intravenous route. A 30-ml fasting blood sample was collected into disodium EDTA (1.0 mg/ml) after 10 min and subsequently at daily intervals for the next 12 days. The plasma from each sample was subjected to rate zonal ultracentrifugation as described above to isolate HDL$_2$ and HDL$_3$. These were dialyzed exhaustively against distilled water, lyophilized, and delipidated (ether–ethanol 3:1, followed by ether). The protein residue was dried under nitrogen and dissolved in 2.0 ml of 5.4 M urea/0.05 M barbital buffer, pH 8.6. The radioactivity in each fraction was determined in a Packard Autogamma spectrometer and its apoA-I content was measured by electroimmunoassay as described below. From these data, the specific activities of ^{125}I- and ^{131}I-apoA-I in HDL$_2$ and HDL$_3$ were calculated.

ApoA-I electroimmunoassay

The method of Laurell (21) was used. Two ml of a 5.4 M urea–0.05 M sodium barbital buffer, pH 8.6, was added to 0.1 ml of each sample to be assayed and the apoA-I content of 5 μl of this mixture was measured by electrophoresis (25 V/cm, 16 hr, 10°C) into a 0.1 × 10 × 10 cm gel of 1% agarose containing 2% anti-apoA-I antibody. ApoA-I standards, dissolved in the same buffer, were also applied to each plate. The assay was linear over the range 25–130 μg apoA-I/ml ($r < 0.996$). The within and between batch coefficients of variation were 3.6% (n = 15) and 4.8% (n = 19) respectively.

In vitro interactions of apoA-II and apoC in HDL subfractions

ApoA-II interactions. Exchange of apoA-II between HDL$_2$ and HDL$_3$ was studied as follows: 3.3 nmol of ^{131}I-apoA-II/HDL$_2$ were incubated for 30 min at 37°C with an equimolar amount of unlabeled HDL$_3$ in a total volume of 8.0 ml of 0.05 M sodium barbital buffer, pH 8.6, and the subfractions were subsequently separated by rate zonal ultracentrifugation. The rotor effluent was collected into 15-ml fractions and the radioactivity was measured in 2-ml aliquots from each fraction. Since ^{131}I was used, no correction was required for salt quenching (22). The experiment was repeated using ^{131}I-apoA-II/HDL$_3$ and unlabeled HDL$_2$.

ApoC interactions. Ten nmol of ^{131}I-apoC/HDL$_2$ or ^{131}I-apoC/HDL$_3$ were incubated in 8.0 ml of barbital buffer, pH 8.6, for 30 min at 37°C with equimolar amounts of unlabeled HDL$_3$ and HDL$_2$, respectively, and the HDL subfractions were separated by rate zonal ultracentrifugation. The distribution of radioactivity through the gradient was determined as described above.

Duplicate in vitro incubation studies gave essentially the same results.

RESULTS

This study was devised to determine the utility of labeled HDL apoproteins, incorporated in vitro into HDL$_2$ and HDL$_3$, as markers of HDL subfraction metabolism. In a previous investigation (23) we showed that ^{125}I-apoA-I binds specifically to HDL in vitro, and at low apoA-I/HDL molar ratios (1/10 or less, assuming an HDL apoprotein mass of 125,000 daltons) there is a mole for mole exchange of exogenous with endogenous apoA-I on the lipoprotein particle. We also found that no more than 2/3 of the endogenous apoA-I participated in this exchange. This report extends our observations relative to the dynamic interactions of this displaceable apoprotein with HDL$_2$ and HDL$_3$.

ApoA-I interactions with HDL$_2$ and HDL$_3$

When radiolabeled apoA-I was incubated separately with HDL$_2$ and HDL$_3$, it became incorporated into each lipoprotein particle (88% of ^{125}I-apoA-I was found in HDL$_2$ and 97% of ^{131}I-apoA-I in HDL$_3$ at an apoprotein/lipoprotein molar ratio of 1/20 in the incubation mixture). The association with the subspecies was maintained during rate zonal ultracentrifugation (**Fig. 2**) and less than 20% (16% from HDL$_2$; 9% from HDL$_3$) of the radioactivity was stripped from the particles by this procedure. Moreover, the flotation properties of the HDL subfractions in the zonal rotor were unaltered by apoA-I binding under the above conditions.

When ^{125}I-apoA-I/HDL$_2$ and ^{131}I-apoA-I/HDL$_3$ were injected simultaneously into the bloodstream of

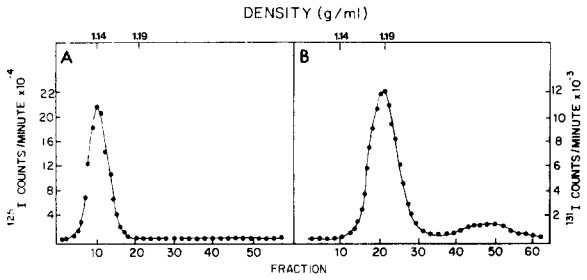

Fig. 2. Binding of apoA-I to HDL_2 and HDL_3. 1.0 nmol of ^{125}I-apoA-I and ^{131}I-apoA-I were incubated for 30 min at 25°C with a 20-fold molar excess of HDL_2 and HDL_3, respectively, and then subjected to ultracentrifugation at d = 1.225 g/ml to separate free from lipoprotein-bound apoprotein. The buoyant HDL subfractions, with their associated radioiodinated apoA-I, were carefully aspirated from the centrifuge tube, dialyzed against 0.05 M barbital buffer, and subjected separately to rate zonal ultracentrifugation. Panel A, ^{125}I-apoA-I/HDL_2; Panel B, ^{131}I-apoA-I/HDL_3.

a healthy male volunteer, a rapid exchange of the labeled apoprotein between the HDL subfractions was observed so that, within 10 min, approximately 10% of the total ^{125}I- and ^{131}I-apoA-I were found in the HDL_2 fraction, and the remainder in HDL_3 (**Fig. 3**), consistent with the relative HDL subfraction distribution in the subject. This transfer of apoA-I between HDL_2 and HDL_3 also resulted in rapid equalization of apoA-I specific activities in the particles. Thus, within 10 min ^{125}I-apoA-I and ^{131}I-apoA-I, injected in association with HDL_2 and HDL_3, respectively, had equilibrated with unlabeled apoA-I in both particles (**Fig. 4**); and the rate of decrease of apoA-I specific activity with time was identical for both particles and isotopes (t½ of ^{125}I-apoA-I and

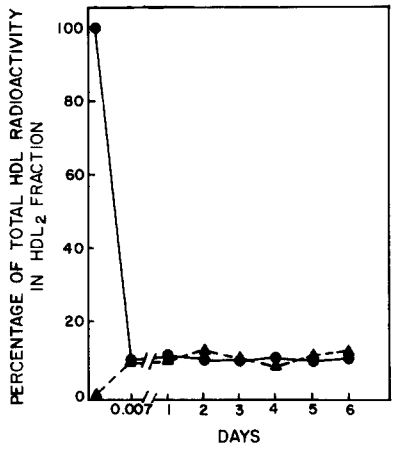

Fig. 3. 25 μCi ^{125}I-apoA-I/HDL_2 and ^{131}I-apoA-I/HDL_3, prepared by in vitro incubation as described in Methods, were mixed, sterilized, and injected into the bloodstream of a healthy male volunteer. The distribution of ^{125}I- and ^{131}I-radioactivity between HDL_2 and HDL_3 was measured at daily intervals using rate zonal ultracentrifugation. (●) ^{125}I radioactivity, injected as ^{125}I-apoA-I/HDL_2; (▲) ^{131}I radioactivity, injected as ^{131}I-apoA-I/HDL_3.

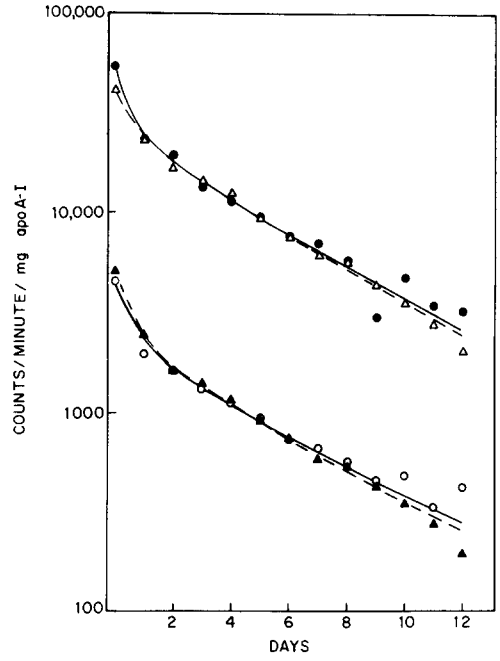

Fig. 4. Specific activity decay curves of ^{125}I-apoA-I and ^{131}I-apoA-I in HDL_2 and HDL_3. The ^{125}I- and ^{131}I-specific activity of apoA-I in the HDL_2 and HDL_3 fractions isolated as described in the legend of Figure 3 was measured at daily intervals by radioactivity determination and apoA-I electroimmunoassay. (●) ^{125}I-apoA-I specific activity in HDL_2; (△) ^{125}I-apoA-I specific activity in HDL_3; (○) ^{131}I-apoA-I specific activity in HDL_2; (▲) ^{131}I-apoA-I specific activity in HDL_3.

^{131}I-apoA-I were 3.7 and 3.75 days, respectively, in keeping with the findings of other workers relative to apo HDL (24, 25)). From this, we concluded that radioiodinated apoA-I, bound to HDL_2 and HDL_3 by in vitro incubation, is rapidly and freely exchangeable between the particles. Consequently, radiolabeled apoA-I is an unsuitable probe for metabolic studies of either particle.

Interactions of apoA-II with HDL_2 and HDL_3

When ^{131}I-apoA-II was incubated with postprandial plasma, 94.5% of the radioactivity was recovered by flotation in the plasma lipoprotein fraction (d < 1.225 g/ml). Agarose gel chromatography of this material showed that 99.6% of the radioactivity was associated with HDL, 0.36% with LDL and 0.04% with VLDL/chylomicra (**Fig. 5**). ^{131}I-apoA-II was incorporated equally well into HDL_2 and HDL_3 in vitro. Ultracentrifugation of the incubation mixture showed that 95.6% and 96.5% of the apoprotein was associated with the respective lipoprotein particles. These labeled lipoproteins, when subjected to rate zonal ultracentrifugation, retained their integrity and flotation characteristics (**Fig. 6A** and **B**). When ^{131}I-apo-II/HDL_2 and ^{131}apoA-II/HDL_3 were incubated with unlabeled HDL_3 and HDL_2 respectively, apoA-II

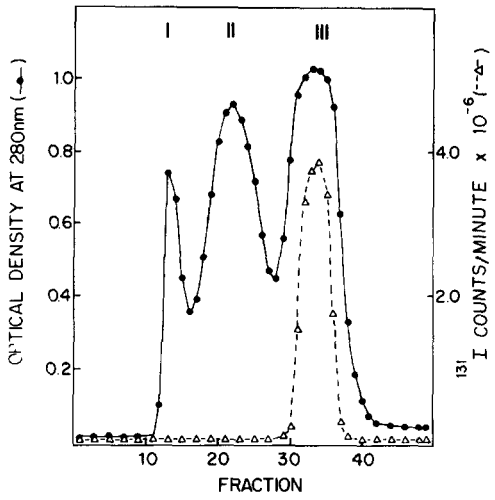

Fig. 5. Apolipoprotein A-II binding to plasma lipoproteins. 1.0 nmol of ^{131}I-apoA-II was incubated for 30 min at 25°C with 6.0 ml of postprandial plasma. Total lipoproteins were then separated from the other plasma proteins by ultracentrifugation at d = 1.225 g/ml, aspirated carefully from the ultracentrifugation tube, and concentrated in a Minicon B15 concentrating cell (Amicon Corp., Lexington, MA). The lipoproteins were fractionated on a column of 6% agarose (Bio-Gel A5m, 200–400 mesh, Bio-Rad Laboratories, Richmond, CA). The absorbance (280 nm) and radioactivity elution profiles are shown. Peaks I, II, and III correspond to VLDL, LDL, and HDL, respectively.

exchange occurred between the two particles (Fig. 6C and D).

Transfer of apoC between HDL subfractions

When ^{131}I-VLDL was incubated with HDL_2, HDL_3 or total HDL and then subjected to ultracentrifugation at a density of 1.020 g/ml, 25–38% of the total radioactivity formerly found in the VLDL fraction became associated with HDL. Ether–ethanol extraction showed that 7.6, 8.8, and 11.4% of the radioactivity displaced from VLDL by total HDL, HDL_2 and HDL_3, respectively was in lipid; more than 90% of the remainder was in the C proteins as demonstrated by polyacrylamide gel electrophoresis. Loss of radioactivity from VLDL did not occur in the absence of HDL, but increased progressively on incubation with equimolar amounts of HDL_3, total HDL, and HDL_2 (25.3%, 33.4%, and 38.4% of total VLDL radioactivity was displaced by HDL_3, total HDL, and HDL_2, respectively). The radioactivity lost from the VLDL appeared in HDL. (**Fig. 7A** and **B**). The extent of transfer of total VLDL apoC was dependent on the HDL subfraction in the incubation; 50% of the labeled apoC in VLDL was transferred to HDL_3 while the transfer to an equimolar amount of total HDL or HDL_2 was 69% and 78%, respectively.

Fig. 7C and D demonstrate that transfer of apoC occurs between HDL_2 and HDL_3. Incubation of ^{131}I-

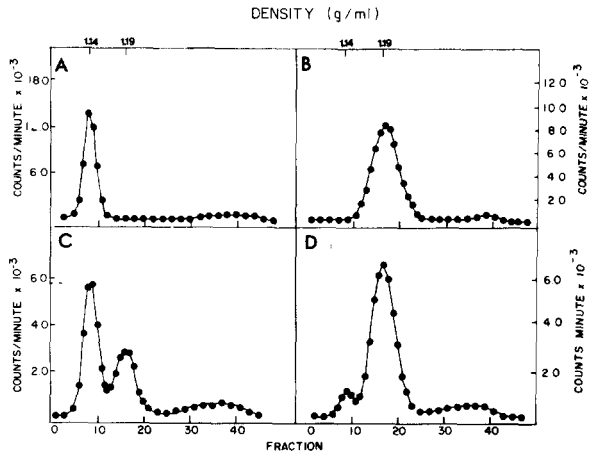

Fig. 6. Interactions of apoA-II with HDL_2 and HDL_3. One nanomol of ^{131}I-apoA-II was incubated with a 20-fold molar excess of HDL_2 or HDL_3 and then subjected to ultracentrifugation at d = 1.225 g/ml to separate bound from free apoprotein. The ^{131}I-apoA-II/HDL subfractions were aspirated carefully from their respective centrifuge tubes, dialyzed separately against 0.05 M barbital buffer, pH 8.6, and their flotation characteristics were checked by rate zonal ultracentrifugation. Panel A, ^{131}I-apoA-II/HDL_2 subjected to rate zonal ultracentrifugation; Panel B, ^{131}I-apoA-II/HDL_3 subjected to rate zonal ultracentrifugation. Exchange of apoA-II between HDL_2 and HDL_3 was detected by incubating the ^{131}I-apoA-II-labeled HDL subfractions with their unlabeled counterparts. The products of incubation were separated by rate zonal ultracentrifugation. Panel C, ^{131}I-apoA-II/HDL_2 + unlabeled HDL_3; Panel D, ^{131}I-apoA-II/HDL_3 + unlabeled HDL_2.

apoC/HDL_2 or ^{131}I-apoC/HDL_3 with equimolar amounts of unlabeled HDL_3 and HDL_2 resulted in exchange of radioactivity between the labeled and unlabeled subfractions. Again, on a molar basis, HDL_2 exhibited

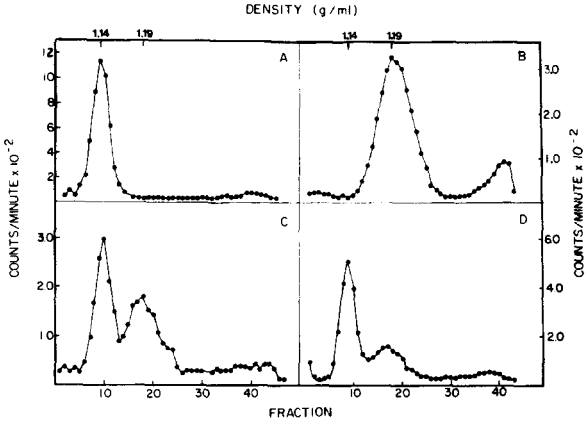

Fig. 7. Transfer of apoC between HDL_2 and HDL_3. The HDL subfractions, labeled with ^{131}I-apoC by transfer from VLDL, were subjected to rate zonal ultracentrifugation. Panel A, ^{131}I-apoC/HDL_2; Panel B, ^{131}I-apoC/HDL_3. Exchange of apoC between the HDL subfractions was examined by incubating ^{131}I-apoC/HDL_2 with unlabeled HDL_3 and vice versa. The subfractions were then separated by rate zonal ultracentrifugation. Panel C, ^{131}I-apoC/HDL_2 + unlabeled HDL_3; Panel D, ^{131}I-apoC/HDL_3 + unlabeled HDL_2.

preferential uptake of apoC. This free exchange of apoC between the HDL subfractions precludes the use of this apoprotein family as a marker for HDL subfraction metabolism.

DISCUSSION

The metabolic interrelationships between the HDL subspecies have for some time presented an intriguing problem whose elucidation has been impeded by the absence of a rapid and reliable means of preparative HDL subfractionation. Application of rate zonal ultracentrifugation to this problem has largely resolved the difficulty, and in this study we have used the technique to search for a probe suitable for the independent measurement of HDL_2 and HDL_3 turnover. Further subfractionation of the lipoprotein was not attempted because of the difficulties involved in obtaining the less well characterized species in pure form. Since HDL lipids exist in a state of continuous flux in the plasma (26), we determined at the outset to examine the interaction of the three major HDL apoproteins (apoA-I, apoA-II, and C proteins) with HDL_2 and HDL_3. Our observations indicate that these, like HDL lipids, are distributed in dynamic equilibrium between HDL_2 and HDL_3. Using different techniques, Grow and Fried (27) have reached the same conclusions. Since the HDL subfractions contain essentially the same lipid and protein components (28), these findings have important structural and metabolic implications. Co-existence of the particles indicates that their constituents can be arranged in two thermodynamically stable forms that are not directly interconvertible, but that freely exchange components. The intrinsic properties of these subfractions that permit their separate existence must also allow for variation of their relative proportions to account for the reported changes in the HDL_2/HDL_3 ratio produced by certain dietary (7) and pharmacologic (8, 29) stimuli. The mechanism whereby these changes are effected is not yet understood, but we believe that it may involve modification of the chemical composition or physical properties of one or both particles, leading to a change in their relative stabilities. Since there is a reciprocal relationship between the HDL_2/HDL_3 ratio and plasma VLDL (those factors that increase the ratio reduce the VLDL concentration and vice versa) it is tempting to speculate that a change in VLDL catabolism with selective transfer of apoC between VLDL and HDL_2 (Fig. 7), originally observed by Havel et al. (30), may initiate the change in the HDL subfraction ratio. Further study is required to evaluate this possibility.

Finally, because of the rapid exchange of apoproteins between HDL subfractions, investigations of HDL subfraction metabolism using labeled apoproteins is not possible.

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