Interrelationship between Apoproteins of Very Low Density Lipoprotein and Other Serum Lipoproteins in Lactating Goats

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ABSTRACT
Lipoproteins of goat serum were labeled in vivo by intravenous injection of tritium labeled DL-lysine monohydrochloride. The specific radioactivity of the very low density plus intermediate density fractions reached a maximum at 1.2 h, declined sharply until about 9 h, and then more slowly. The specific radioactivities of the low density and high density lipoproteins reached lower maxima at 6.5 h and 8.5 h, respectively.

When iodine-125-labeled very low density lipoprotein of goats was incubated in vitro with buffer there was a transfer of 11% of radioactivity, chiefly to the intermediate and low density lipoproteins, but on incubation in serum 29% of the radioactivity was distributed equally among the other lipoproteins. On gradient gel electrophoresis of the apolipoproteins, the slowest migrating zones in the very low density, intermediate density, and low density lipoproteins were the most radioactive whereas over 90% of the radioactivity in the apoproteins of the high density lipoprotein was in two zones, one of which was at the origin.

The results are consistent with the formation of low density lipoprotein from very low density lipoprotein via the intermediate density lipoprotein and with a transfer of apoproteins between very low density and high density lipoproteins.

INTRODUCTION
In dairy cows certain lipoproteins in serum transport triglycerides to the mammary gland for synthesis of milk fat (19), but it is not known whether the lipoproteins are interconvertible as are the lipoproteins of humans and rats (12) or, if so, which of their constituent apoproteins are transferred.

The spectrum of lipoproteins is simpler in goat than in cow (5) and when, in connection with another study, a supply of goats' milk containing 3H-labeled protein was required, the opportunity was taken to study the turnover of lipoproteins of goat serum.

The resultant specific radioactivity-time curves were complex and to reach a better understanding of them, 125I-labeled VLDL was incubated in buffer and in serum, and the distribution of radioactivity in the constituent apoproteins of the serum lipoproteins was studied.

MATERIALS AND METHODS

In Vivo Experiments
A British Saanan goat 7.5 yr old in the 18th mo of lactation giving 4 to 4.5 kg milk per day and fed on hay and concentrates (principally rolled barley and flaked maize plus mineral and vitamin supplements) to appetite was used. In the first experiment, 10 mCi of DL-[4,5-3H(n)]-lysine monohydrochloride (Radiochemical Centre, Amersham, England) was administered. There was a little difficulty with the intravenous injection and 3 mCi were injected subcutaneously and 7 mCi via a cannula in the jugular vein. Seven days later 10 mCi of 3H-labeled mixed amino acids were administered via the jugular cannula, and milk samples only were received October 21, 1976.

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2 Abbreviations: very low density lipoprotein, VLDL, d<1.006 g/ml; intermediate density lipoprotein, IDL, 1.006<d<1.019 g/ml; low density lipoprotein, LDL, 1.019<d<1.06 g/ml; high density lipoprotein, HDL, 1.06<d<1.21 g/ml; disintegrations per minute, dpm; counts per minute, cpm.

obtained. Thirty-six days after the second radioactive dose, 20 mCi of DL-[3H]lysine HCl were administered via the cannula (second experiment). Blood samples were obtained via a cannula in the jugular vein opposite to that in which the labeled lysine was introduced and from a cannula in the mammary vein.

Dextran sulfate reagent (5) consisted of a mixture of 1 vol of 10% (wt/vol) clinical grade dextran sulfate (Mw ca. 5000) (Glaxo Laboratories Ltd., Greenford, Middx, U.K.) and 5 vol of 1M CaCl₂ solution. The optimum volume of reagent required to precipitate the serum lipoproteins of density less than 1.06 g/ml (.15 ml reagent/ml serum) was found by titration of small aliquots of serum with the reagent to maximum turbidity at 700 nm. The appropriate volume of reagent was added either to 10 ml serum (Experiment 1) or to 20 ml serum (Experiment 2). The precipitate was dissolved in sodium citrate solution (d = 1.019 g/ml) containing 1 mg/ml DL-lysine HCl and reprecipitated with .1 M CaCl₂ solution (5). Each sample was reprecipitated three times to reduce the free [3H]lysine content to negligible proportions. The precipitate then was dissolved in sodium citrate (6 ml, d = 1.019 g/ml), transferred to a polycarbonate centrifuge tube (17 mm x 63 mm, nominal capacity 10 ml), and the VLDL + IDL and LDL were separated by ultracentrifugation at 157,000 x g (av) for 20 h at 12 C in the 10 × 10 ml angle rotor (tube angle 20° to axis) of an MSE Superspeed Ultracentrifuge (29).

After removal of the precipitable lipoproteins, the HDL was obtained by ultracentrifugation of the supernatant, under the above conditions, after its density had been adjusted to 1.21 g/ml by the addition of solid KBr. The HDL was purified by further ultracentrifugation at the same density.

To remove free [3H]lysine, the HDL samples were dialyzed overnight against running distilled water and then against three batches of distilled water on three successive nights. The purified lipoproteins were stored in sodium citrate solution (d = 1.019 g/ml), and their purity was checked by electrophoresis (5). The protein contents were determined according to Lowry et al. (21) but with the addition of .1 ml of a 5% (vol/vol) Teepol solution to the VLDL + IDL assay mixtures to remove the turbidity which developed.

Radioactivity was measured as in (2) in the scintillator solution containing methoxyethanol-anisole; 10 ml of scintillator solution were mixed with .6 ml (Experiment 1, 13% counting efficiency) or 1 ml (Experiment 2, 11% counting efficiency) of lipoprotein solution.

The radioactivities of the lipoproteins/mg protein, i.e., the specific radioactivities of the apoproteins, were plotted semilogarithmically against time. In Experiment 2, the specific radioactivities were corrected for the low initial specific radioactivity which resulted from the earlier injections. For VLDL + IDL (Experiment 2), the points after the period of rapid decline in specific radioactivity which followed the maximum were fitted to a straight line by the method of least squares.

In Vitro Experiments

Serum was prepared from jugular blood withdrawn from lactating Saanen goats which were fed on hay and concentrates (principally barley, maize, and wheat bran plus vitamin and mineral supplements) to appetite. The blood samples were allowed to clot for 2 h at room temperature and centrifuged then at 2000 rpm for 20 min. Disodium ethylenediamine tetraacetate (EDTA) was added to serum to give a final concentration of .1%.

Chylomicrons were removed from the serum by overlayering 6 ml of serum with 2 ml of a solution of density 1.006 g/ml (sodium citrate or saline) and were centrifuged for 30 min at 60,000 × g (av). Lipoprotein fractions were separated by sequential ultracentrifugation of 8 ml of chylomicron-free serum at increasing densities in the 50 Ti rotor of a Beckman L2-50 preparative ultracentrifuge at 14 C. Solid sodium citrate was used to obtain the required densities.

Four lipoprotein fractions were isolated at densities of 1.006 g/ml (VLDL), 1.019 g/ml (IDL), 1.06 g/ml (LDL), and 1.21 g/ml (HDL). The VLDL and IDL were separated after 18 h and LDL and HDL after 24 h of centrifugation at 105,000 × g (av). Each fraction was brought to 8 ml with sodium citrate solution of its respective density and recentrifuged for 24 h. The purity of each fraction was checked by electrophoresis (5).

The VLDL were iodinated by a modification
of the iodine monochloride method of McFarlane (22) as described by Reif (26). The Na\(^{125}\)I was obtained from the Radiochemical Centre, Amersham, England. Unreacted iodide was removed by dialysis against 8 to 10 changes of .15 M NaCl and .01% EDTA, for 6 to 7 h at 4 C. The efficiency of labeling of the VLDL with \(^{125}\)I was 25.85 ± 3.1%, and protein recovery after iodination was 86.3 ± 8.7% (means of three values ± SD). The ratio of moles of iodide to 100,000 g of apoprotein was less than 1 in all preparations as recommended by Eisenberg and Rachmilewitz (13). Radioactivity was determined in the Autogamma Scintillation Spectrometer (Packard Tri-carb).

The labeled VLDL was incubated with serum for 1 h at 37 C. In the control, labeled VLDL was incubated with .15 M NaCl in .05 M phosphate buffer, pH 7.6 under the same conditions. Each determination was in duplicate. Aliquot samples then were withdrawn for \(^{125}\)I monitoring. The lipoprotein fractions subsequently were isolated from the remaining samples by centrifuging at their respective densities. The \(^{125}\)I content in each fraction was measured. The fractionated samples were lyophilized and delipidated as described by Brown, Levy and Fredrickson (3), and the \(^{125}\)I content of the lipid fraction was determined. The apoproteins were solubilized by incubation (37 C for 16 h) in .02 M tris/HCl (pH 8.2) containing .1 M sodium dodecylsulfate and .001 M dithiothreitol, diluted 1:1 (vol/vol) with 40% (wt/vol) sucrose before gel application, and fractionated by electrophoresis on 5 to 11% polyacrylamide gradient gels containing 1% sodium dodecylsulfate. Bromophenol blue was used as a marker (7). The gels were stained with .05% Coomassie Blue R in 12.5% (wt/vol) trichloroacetic acid (6), destained in a solution of water to ethanol to acetic acid (65:25:8 by vol), (31), and sliced into zones whose radioactivities were determined.

**RESULTS**

**In Vivo Experiments**

Figure 1 shows that there was no cross contamination between the precipitable and nonprecipitable lipoproteins. In addition, it has been established (Stead and Welch unpublished) that when lipoproteins of goat serum are isolated by means of dextran sulfate as described here, the LDL has the same electrophoretic mobility as, and is immunologically identical to, LDL isolated by ultracentrifugation alone. Furthermore, the nonprecipitable lipoprotein is immunologically identical to HDL isolated by ultracentrifugation, has the same flotation rate in the analytical ultracentrifuge, and does not contain LDL.

The specific radioactivities of the apoproteins of the lipoproteins from the second experiment are plotted semilogarithmically against time in Figure 2a; the values for jugular and mammary venous serum appeared to be the same, and they were treated as equivalent when the curves were drawn. The maximum value for the specific radioactivity of the VLDL + IDL apoprotein was 22 dpm/\(\mu\)g, which was reached 1.2 h after injection, whereas that of LDL was 7.1 dpm/\(\mu\)g at 6.5 h, and that of HDL 2.8 dpm/\(\mu\)g at 8.5 h.

The decline in specific radioactivity did not follow a simple exponential curve for any of the lipoproteins. In particular, there was a marked change in the slope of the VLDL curve at about 9 h, which indicated that the curve represented apoproteins with different kinetic behavior. The specific radioactivity-time curves
FIG. 2. Specific radioactivities of the protein moieties of goat very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) after administration of DL-[\(^{3}H\)]lysine monohydrochloride. (a) Experiment 2; (b) Experiment 1. *, jugular venous serum; ▲, mammary venous serum.

which were obtained in the first experiment were similar to those of the second experiment in shape, and in time and relative size of the maximum (Fig. 2b).

In Vitro Experiment

The protein concentrations of the lipoprotein fractions of goat serum [expressed as mg/100 ml ± SD (means of six determinations)] were: VLDL, 1.28 ± .12; IDL, 2.1 ± .3; LDL, 8.1 ± 1.8; HDL, 135.8 ± 7.6. The distribution of radioactivity among the four lipoprotein classes after incubation of the \(^{125}I\)-labeled VLDL with serum and phosphate/sodium chloride buffer is in Table 1.

Approximately 80% of the total radioactivity in the lipoproteins recovered after incubation with either serum or buffer was bound to proteins. On incubation of the labeled VLDL with serum, 29% of the radioactivity originally in this fraction was transferred to other lipoproteins and was distributed approximately equally among the other three lipoprotein fractions. On incubation with buffer, however, only 11% of the radioactivity originally in the VLDL fraction was recovered in other lipoproteins, and there was approximately twice as much radioactivity in the IDL and LDL as in the HDL.

After delipidation of the lipoproteins, the following proportions of radioactive protein were resolubilized: VLDL, 70%; IDL, 90%; LDL, 75%; HDL, 98%. The electrophoretic patterns of the apoproteins are shown diagrammatically in Figure 3. The distribution of radioactivity among the apoproteins after incubation of \(^{125}I\)-labeled VLDL in serum is in Table 2. The distribution of radioactivity was similar in the VLDL, IDL, LDL; the most radioactive bands were in zones 1 and 2.

In the VLDL, zone 3 was also radioactive,

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<tr>
<th>TABLE 1. Redistribution of (^{125}I) from very low density lipoprotein to other lipoprotein fractions.(^{a})</th>
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<td><strong>Incubation medium</strong></td>
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<td>Lipoprotein fraction</td>
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\(^{a}\) Each value is the mean of four determinations.
but although the most intense band in the LDL was in this zone, it was not radioactive. In the HDL over 90% of the radioactivity was concentrated in two bands in zones 1 and 6.

Zone 6 of the VLDL was the zone from which the greatest proportion of radioactivity was lost on incubation in serum as compared to incubation in buffer.

**DISCUSSION**

Human and rat VLDL contain apoproteins which are common to LDL (apoprotein B) and to HDL (apoprotein C). The apoprotein B of VLDL is converted rapidly in vivo to apoprotein B of the LDL via the formation of a lipoprotein of intermediate density (12, 17). This transfer does not take place to any great extent when labeled VLDL is incubated in serum for 20 to 30 min (1, 27), but when the incubation time is increased, and particularly when post-heparin plasma is used, there is an appreciable transfer (8). The apoprotein C of VLDL exchanges with the apoprotein C of the HDL in vitro and in vivo (11, 12), and there is also a net transfer of apoprotein C to HDL in rats in vivo and when VLDL is incubated in post-heparin plasma (15). The different phases of the complex disappearance curve of VLDL radioactivity after injection of $^{125}$I-labeled VLDL (13) can be attributed to differences in the rates of turnover of the VLDL apoproteins (11, 14).

The specific radioactivity-time curves for the apoproteins of goat serum after an injection of DL-$[^3]$H]lysine were similar to those for human apoproteins after injection of $[^75]$Se]selenomethionine (9, 28) and for five species of animals after injection of DL- or L-$[^3]$H]lysine (18). The early maximum and sharp fall in specific radioactivity of VLDL + IDL apoproteins of goat were consistent with the precursor-product relationship which has been suggested in humans between the apoprotein B of VLDL and that of LDL (8, 9, 28). In support of this, most of the radioactivity in the IDL and LDL of goat serum, after incubation with $^{125}$I-labeled VLDL, was in electrophoretic zones 1 and 2. In 10% polyacrylamide gels, apoprotein B remained at the origin (12), and although direct comparison is not possible because the electrophoretic system in our work was different, it seems likely that zones 1 and 2 could correspond to apoprotein B. Similarities between the apoproteins of VLDL and LDL$_2$ (1.039 < d < 1.060 g/ml) have been demonstrated also in cows (30).

The complex decay curve of the specific radioactivity of VLDL + IDL apoprotein of goats was also similar to that of VLDL radioactivity in rats after injection of $^{125}$I-labeled VLDL (13). The slower turning over component of the decay curve of rat $^{125}$I-labeled VLDL (13) was attributed to the turnover of apoprotein C which equilibrates between VLDL and HDL (12, 14). In our experiments more radioactivity transferred from $^{125}$I-labeled VLDL of goat to other lipoproteins when it was incubated with serum than could be accounted for by control incubation in buffer (Table 1), and the relative increase in radioactivity was greatest in HDL (Table 1). On electrophoresis of apoproteins 42% of HDL radioactivity was recovered in zone 6 (Table 2). After incubation, zone 6 of the HDL contained more radioactivity than was recovered altogether from the corresponding zones of other lipoproteins, and this zone of the VLDL was the one which lost the greatest proportion of radioactivity after incubation in serum. It seems probable, therefore, that zone 6 corresponds to apoprotein C which exchanges between VLDL and HDL in humans (1, 10, 16) and in rats (14, 15, 27). Apart from transfer of apoprotein...
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<tr>
<th>Lipoprotein fraction</th>
<th>Zone$^b$</th>
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<th>2</th>
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<td>18.9</td>
<td>29.9</td>
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<td>a)</td>
<td>3.4</td>
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| Incubation with buffer |       | 12.5 | 32.9 | 10.9 | a) | 9.0 | 6.0 | 7.5 | a) | 3.5 | a) | 1.0 | .8 |
|                       |       |      |      |      | b) | 5.5 |     |     | b) | 2.4 | b) | 6.5 |

$^a$Values are percent of total radioactivity recovered/gel.

$^b$Zones are as in Figure 3.
from VLDL to LDL and HDL in vivo, direct synthesis of LDL and HDL (18, 20), and recycling (9, 23), possibly may occur.

In cows, whose lipoproteins have been studied more extensively than those of the goat, apoproteins of VLDL (d<1.019 g/ml) and HDL have similarities (30). An additional lipoprotein, LDL1 (1.019<d<1.039 g/ml) with an electrophoretic mobility similar to that of HDL, is sometimes present but has not been observed in sheep and goats (5). The LDL1 is immunologically related to HDL (30) and like HDL can activate milk lipoprotein lipase (4). Its concentration is variable, greater in lactating cows than in dry cows (24, 25), and is increased in the blood of cows fed protected fat (Stead and Welch unpublished).

Apoprotein C regulates the activity of lipoprotein lipase (12), and if it is in bovine VLDL and HDL (as appears to be the case in the goat) and, therefore, possibly in LDL1, but not in LDL2 [human LDL normally contains only traces of apoprotein C (12)], this could explain why bovine VLDL and LDL1 supply triglycerides for milk fat synthesis whereas LDL2 may not (19). It does not explain why bovine HDL triglyceride is not a precursor of milk fat and this together with a study of the factors which affect the concentration of the bovine lipoproteins and their interconversion is under investigation.

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