Organization of the Core Lipids of High Density Lipoproteins in the Lactating Bovine*

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During lactation, cows develop greatly increased levels of plasma high density lipoproteins (HDL), including a population of particles of larger size than normal human plasma HDL. To study the relationship between lipoprotein size and organization of their core lipids, HDL from lactating Jersey and Holstein cows were fractionated by isopycnic density gradient ultracentrifugation, and individual fractions by densities 1.06 and 1.13 g/ml were examined by differential scanning calorimetry and negative stain electron microscopy. With increasing density, particles decreased in mean diameter from 16.0 to 9.0 nm. Scanning calorimetry of HDL of diameters 12.0 to 16.0 nm showed a reversible thermal transition between 28 and 35°C, resembling the liquid crystalline transitions of cholesterol esters in the lipid extract of bovine HDL. The enthalpy of this transition was markedly reduced compared to pure cholesterol esters or to the cholesterol ester transitions associated with the appearance at high temperatures of cholesterol esters or to the cholesterol ester transitions associated with the appearance at high temperatures of cholesterol esters observed in HDL of density 1.06 to 1.12 g/ml (11-13). Such large HDL particles are normally absent from human plasma or present in low concentration (13). Bovine HDL of density 1.06 to 1.12 g/ml have diameters from 12 to 16 nm, intermediate between human HDL and swine HDL. They therefore offer the opportunity to test the hypothesis that there is a critical change in the thermal behavior of the lipoprotein core in this size region. We have isolated HDL from lactating cows by density gradient ultracentrifugation and examined the individual fractions by differential scanning calorimetry and x-ray scattering. The results show a markedly perturbed order-disorder transition of cholesterol esters present only in the larger bovine HDL particles.

MATERIALS AND METHODS

Four nonpregnant lactating cows ( Jersey and 3 Holsteins) were used for this study. Blood was drawn from the jugular vein into bags containing acid/citrate/dextrose and the plasma was recovered by centrifugation at 4°C. The plasma and all salt solutions used for centrifugation contained Na2EDTA (0.04%), Na3citrate (0.08%), and gentamicin (0.005%). Solution densities were determined using a Mettler/Toledo Densiometer (Graz, Austria). Lipoproteins were prepared by sequential preparative ultracentrifugation using a Beckman 40.3 rotor at 39,000 rpm and 16°C. Cellulose nitrate tubes were filled with 5 ml of plasma. The d < 1.006 and the 1.006 to 1.050 g/ml fractions were pipetted from the top 2 ml following separate 18-h periods of centrifugation. The 1.050 to 1.21 g/ml fractions were recovered in the top 1 ml in a similar manner after 24 h of centrifugation. In each case, the infranatants were adjusted to the higher density by the addition of NaCl/NaBr solution according to the methods of Lindgren (14).

For preparation of density gradients, separate 2-ml aliquots of a pool of the 1.050 to 1.21 g/ml fraction were transferred to cellulose nitrate tubes (1.4 x 8.9 cm). These were overlaid with a mixture of 4 ml of the 1.050 to 1.21 g/ml fraction pool and 2 ml of 0.196 M NaCl solution. The tubes were filled with 4.5 ml of a NaCl/NaBr overlaying solution (d = 1.050). Following 40 h of centrifugation at 37,000 rpm and 16°C in a Beckman SW 41 rotor, 1 ml fractions were

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X-ray scattering and electron microscope studies have shown that plasma lipoproteins are quasispherical particles in which a core of apolar lipid is surrounded by a more polar surface of lipids and apoproteins (1). The plasma low density lipoproteins (density 1.019 to 1.063), the major cholesterol transporting lipoproteins of human plasma, display reversible order-disorder transition of their core cholesterol esters in the vicinity of body temperature (2-8). A similar transition of cholesterol esters was observed in HDL (15 to 19 nm) isolated from swine fed an atherogenic diet (6, 9). By contrast, intact human HDL (diameter 10 to 12 nm) do not display any transition of cholesterol esters between 0 and 60°C (10). These observations suggest that between 15 and 12 nm in diameter there is a critical size change that dictates an alteration in the organization of the lipoprotein core lipids.

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1 The abbreviations used are: HDL, high density lipoproteins; SDS, sodium dodecyl sulfate; DSC, differential scanning calorimetry; LDL, low density lipoproteins; apo, apolipoprotein.
collected from the top of each tube using an ISCO density gradient fractionator (Lincoln, Neb.).

In each fraction, concentrations of total and free cholesterol (15), triglycerides (16) and lipid phosphorus (17) were determined. Protein concentrations were determined by a modified Lowry procedure (18) employing 1% SDS in a Na2CO3 buffer and using bovine albumin as a standard. Polyacrylamide gel electrophoresis was performed in 0.1% SDS-15% acrylamide by a modification (19) of the procedure of Laemmli (20).

Differential scanning calorimetry was performed on HDL samples using a Perkin-Elmer DSC-2 (21, 22). Samples were concentrated by vacuum dialysis prior to DSC experiments, using 0.15 M NaCl, pH 8.0. Seventy-five-micro liter aliquots containing 50 to 150 mg of lipid-protein/ml were sealed hermetically in stainless steel pans and examined at heating rates of 5°C/min and cooling rates of 10°C/min. Baseline curvature in these experiments largely reflects differences in radiative heat loss between the sample and reference pans. Polarized light microscopy, x-ray scattering, and negative stain electron microscopy were performed as described previously (4, 23).

**RESULTS**

Plasma HDL obtained from lactating cows (1 Jersey and 3 Holsteins) was fractionated by isopycnic density gradient ultracentrifugation between 1.05 to 1.20 g/ml. Representative compositional data are shown in Table I, for a Jersey and a Holstein. The greatest concentration of HDL was recovered in fractions of 1.069 to 1.083 g/ml. Increasing density of the isolated fractions was accompanied by increased relative content of protein and a decreased content of cholesterol and cholesterol ester (note that percentage compositions are shown in brackets in Table I). Triglyceride represented less than 1 weight % of the lipoprotein mass, whether determined by fluorometric assay (16) or by quantitative thin layer chromatography (24). The bottom fractions of the gradient contained small amounts of phospholipid and proteins. For all of the HDL fractions, the major apoprotein consisted of a single band with electrophoretic mobility in 15% polyacrylamide SDS gels similar to human apoA-I (Fig. 1), as reported by Jonas for nonlactating bovine HDL (25). The fast migrating bands seen in the gels presumably represent small amounts of apoC-like peptides or reduced apoA-II and were more prominent in the less dense fractions.

Differential scanning calorimetry was performed on each of density fractions 2 to 6 obtained from all four animals. Fraction 1 was not examined because it contained β- as well as α-migrating lipoproteins and fractions 7 to 10 contained insufficient material for calorimetric analysis. The DSC curves obtained from one set of fractions from a lactating Holstein cow are shown in Fig. 2. Lipoproteins from gradient fractions 2 and 3 displayed reversible transitions between 20 and 35°C (Fig. 2, a and b). There was no similar transition in the intact lipoproteins of greater density (Fig. 2, c to e). On heating to higher temperatures, all fractions showed an irreversible denaturation endotherm. Following thermal denaturation, all lipoproteins displayed reversible liquid crystalline transition of cholesterol esters between about 30 to 45°C as exemplified in Fig. 2f. The identity of the 30 to 45°C transition was confirmed by examination of the lipid extract of the bovine HDL by DSC and by polarized light microscopy of heat-denatured HDL as described previously (10). These results indicate that the transition between 20 and 35°C observed in the intact HDL samples was probably due to an order-disorder transition of cholesterol esters. However, the enthalpy of this transition (0.1 to 0.6 cal/g of cholesterol ester) was reduced compared to pure cholesterol esters (1 cal/g) (23), low density lipoproteins (0.7 cal/g of cholesterol esters (4)), and HDL (0.7 to 0.8 cal/g of cholesterol esters (6)).

In Fig. 3 is shown the relationship between lipoprotein diameter and the enthalpy of the order-disorder transition of cholesterol esters. The individual data for the bovine HDL samples and the mean enthalpies for LDL (4) and HDL (6) are shown. The data for LDL and HDL suggest no change in

![FIG. 1. SDS-polyacrylamide gels of proteins isolated from density gradient fractions 2 to 7 of bovine HDL.](image-url)
transition enthalpy between about 16.0 nm in diameter. There is a marked decrease in enthalpy of the order-disorder transition of bovine HDL between about 16 and 12 nm in diameter, and no transition in smaller particles. The sizes of the HDL shown in Fig. 3 are based on negative stain electron micrographs. Diameters of the same fractions determined from quasielastic light scattering measurements were in agreement to within 1.0 nm.

On heating the bovine HDL fractions to higher temperature, there was an irreversible lipoprotein denaturation endotherm (Fig. 2). For fractions which displayed a cholesterol ester transition between 20 and 35°C, the denaturation endotherm consisted of a single peak with a small secondary peak at a slightly higher temperature (Fig. 2, a and b). With increasing density of the lipoproteins, the high temperature component of the denaturation endotherm became more clearly separated and increased in size. The first broad component had a constant peak temperature of 90 ± 1.0°C, while the second component had a peak temperature that increased up to 113°C. The smaller HDL also showed a greater enthalpy of denaturation. Expressed in calories/g of lipoprotein, the total denaturation endotherm increased in enthalpy from 0.67 cal/g (fraction 2) to 1.72 cal/g (fraction 6). The enthalpy and entropy changes associated with the two components of denaturation were calculated in terms of each of the lipoprotein constituents after approximating the area under each peak by extrapolation to baseline (10). The first component of the denaturation endotherm had a constant value only when expressed as an enthalpy change of protein (2.5 cal/g of protein). The second component was not associated with a constant enthalpy change when expressed in terms of any individual lipoprotein component. However, for fractions 4 to 6, where this second component was well resolved, it represented an invariant entropy change for cholesterol esters of approximately 0.0032 cal/g°C. This value is identical to the entropy change associated with the smectic-liquid transition of pure cholesterol esters (0.0032 cal/g°C) (4, 23).

To investigate the identity of the two peaks of the denaturation endotherm of bovine HDL, paired samples were subjected to incremental heating studies in the calorimeter. One sample was heated directly to 130°C while the other was heated to a series of progressively higher temperatures (Figs. 4 and 5). The release of apoA-I was monitored by the appearance of the endotherm of peak temperature 60°C. The latter transition was present in bovine apoHDL and resembles the reversible unfolding endotherm of human apoA-I, except that it is at a slightly higher temperature (22). The release of cholesterol esters was monitored by the appearance of the cholesterol ester liquid crystalline transition between about 20 and 45°C. In Fig. 4, when fraction 6 was heated directly to 130°C it showed two peaks at 90 and 113°C (a). When a second sample was heated to just below the onset of the 90°C peak (b), it showed no release of cholesterol esters or apoA-I. However, further heating through the 90°C peak (c) released apoA-I as shown by the peak at 60°C (d). No cholesterol esters were released, however, until the sample was heated through the 113°C peak (d). Reheating then clearly showed the cholesterol esters as a double peak at 20 to 45°C (e). The results suggest that the first component of the denaturation endotherm was associated with release of apoA-I from the HDL particle, while the second component was associated with release of cholesterol esters and lipoprotein disruption. In the larger HDLs, these components are not well separated and some cholesterol ester is released simultaneously with apoA-I (Fig. 5). However, in the smaller HDLs, it appears that 70 to 80% of apoA-I is released with the first component of the denaturation endotherm without any release of cholesterol esters (Fig. 4).

X-ray scattering was performed on the larger HDL (frac-

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2 S. T. Kunitake, unpublished observations.
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Fig. 4. DSC heating curves of bovine HDL from density gradient fraction 6. In a, the sample (~12 mg of lipoprotein) was heated directly from 0 to 130°C. In b to f, a second sample (~8 mg of lipoprotein) was heated to 78°C, cooled, heated to 102°C, cooled, heated to 130°C, cooled, then heated to 100°C.

Fig. 5. DSC heating curves of bovine HDL from density gradient fraction 5. In a, the sample (~4 mg of lipoprotein) was heated directly from 0 to 120°C. In b to h a second sample (~20 mg of lipoprotein) was heated to progressively higher temperatures as shown.

DISCUSSION

The present investigation is an extension of previous studies of the structure of the core lipids of plasma lipoproteins (2-8). Low density lipoproteins from a variety of species (2-8) and HDL from swine (6, 9) display order-disorder transition of their core cholesterol esters in the vicinity of body temperature. This transition has been attributed to a change of cholesterol esters from a smectic-like or layered state to a more disordered state. The evidence is that, 1) the transition is identical in temperature and enthalpy to the smectic-cholesteric transition of cholesterol esters isolated from the same sample, and 2) below the transition there is a maximum at 1/3.6 nm\(^{-1}\) in the x-ray scattering profile which disappears above the transition (4). An identical fringe is displayed by smectic liquid crystals of pure cholesterol esters (4). Moreover, Fourier transform analysis of the x-ray scattering profiles of both LDL and HDL shows an interior electron density maximum ~4.5 nm from the surface of the particles which arises from a region of overlapping steroid ring moieties (5-7). Assuming the particles have spherical symmetry, these findings can be most readily explained by a model of LDL and HDL containing radially oriented layers of cholesterol ester (5, 6). Luzzati and co-workers (26) have shown that the x-ray scattering profile of LDL below the transition can be attributed to micellar, rather than layered structures of the core cholesterol esters. Provided initial assumptions of nonspherical particle symmetry are made.

In the present study, HDL from the gradient fractions of density 1.06 to 1.09 g/ml displayed reversible transitions between 20 and 35°C (Fig. 2) which probably represent order-disorder transitions of cholesterol esters. This transition, occurred at a similar temperature to the liquid crystalline transitions of the isolated bovine HDL cholesterol esters. Also, in the incremental heating studies, the 20 to 35°C transition in the intact lipoprotein gradually merged with the 30 to 45°C cholesterol ester transition in the heat denatured lipoproteins (Fig. 5). However, in intact HDL the peak temperature of this transition was depressed by about 12°C relative to the smectic-cholesteric transition of released cholesterol esters, and the enthalpy of the transition was reduced compared to pure cholesterol esters, LDL or HDL. In addition, the x-ray scattering profile of these particles did not show the thermotropic changes previously attributed to smectic-disordered transitions of cholesterol esters in LDL and HDL. Thus, our results suggest that the endotherm occurring at 20 to 35°C in intact HDL was due to a transition of cholesterol esters between states of similar enthalpy, probably representing a minor structural rearrangement.
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The thermal denaturation of the smaller bovine HDL particles was accompanied by a characteristic double-peaked endotherm resembling that of humanized swine HDL (10, 6). Previous studies of human HDL have shown that the first broad component of the denaturation endotherm is associated with release of apoA-I and the second sharper component with disruption of the particle and release of apoA-II and cholesterol esters (10). Since the bovine and the swine HDL (6) lack significant amounts of an apoA-II-like protein, the present results show that the second component of the denaturation does not necessarily reflect a conformational change of apoA-II. In both bovine and human HDL, this component was associated with release of cholesterol esters. The entropy change associated with this transition was constant when expressed in terms of cholesterol esters (0.003 cal/g/°C). It is notable that the second peak of the denaturation endotherm of human HDL is also associated with an entropy change of ~0.003 cal/g/°C (10). Thus, the second endotherm may reflect disordering of cholesterol esters associated with particle disruption. When particles of increasing density are compared, this endotherm appears as the low temperature transition of cholesterol esters which cannot melt at low temperatures because of constraints placed on the core by particle size. Following release of most of the apoA-I, these particles are no longer stable and cholesterol esters display order-disorder transitions in association with particle disruption. Although the investigations of HDL by nuclear magnetic resonance spectroscopy have not shown ordered structure of cholesterol esters (27), it may be that such structures cannot be observed on the NMR timescale, as suggested for the boundary lipid of intrinsic membrane proteins (28).

The lactating cow may represent an exaggerated model of the structure and metabolism of HDL in other species. During lipolysis of triglyceride-transporting lipoproteins, there is transfer of surface material (phospholipids, apoA-I, and apoC) into the HDL fraction in man and the rat (29–33). Studies on the uptake of phospholipids and apoproteins by HDL in vitro show that the incorporation of chylomicron or VLDL surface material into HDL may be associated with a decrease in density of the HDL fraction (34, 35). The massive lipolysis of triglyceride-transporting lipoproteins that occurs during lactation in the cow may cause the marked increase in HDL, particularly in the less dense subfractions. Studies of human HDL subfractions isolated by density gradient ultracentrifugation show that the thermal denaturation pattern changes as a function of density in a similar way to bovine HDL, i.e. there is greater thermal stability in the more dense HDL fractions (35). Incorporation of phospholipids into the more dense human HDL subfractions leads to formation of HDL with lower hydrated density and decreased thermal stability (35). The differences in thermal stability of subclasses of bovine HDL may reflect structural alterations associated with acquisition of surface components of lipolyzed triglyceride-transporting lipoproteins.

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REFERENCES
1. Shipley, G. E., Atkinson, D., and Scaru, A. M. (1972) J. Supramol. Struct. 1, 98–104
2. Chen, G. C., and Kane, J. P. (1974) Biochemistry 13, 3336–3335
3. Deckelbaum, R. J., Shipley, G. G., Small, D. M., Lees, R. S., and George, P. K. (1975) Science 190, 392
4. Deckelbaum, R. J., Shipley, G. G., and Small, D. M. (1977) J. Biol. Chem. 252, 744–754
5. Atkinson, D., Deckelbaum, R. J., Small, D. M., and Shipley, G. G. (1977) Proc. Natl. Acad. Sci. U. S. A.
6. Tall, A. R., Atkinson, D., Small, D. M., and Mahley, R. W. (1977) J. Biol. Chem. 252, 7286–7293
7. Tall, A. R., Small, D. M., Atkinson, D., and Rudel, L. L. (1978) J. Clin. Invest. 62, 1354–1363
8. Kirchhausen, T., Unbracht, S. H., Fless, G. M., and Scaru, A. M. (1979) Atherosclerosis 33, 69–76
9. Atkinson, D., Tall, A. R., Small, D. M., and Mahley, R. W. (1978) Biochemistry 17, 3930–3933
10. Tall, A. R., Deckelbaum, R. J., Small, D. M., and Shipley, G. G. (1977) Biochim. Biophys. Acta 487, 145–153
11. Raphael, B. C., Dimick, P. S., and Puppione, D. L. (1973) J. Dairy Sci. 56, 1025–1032
12. Puppione, D. L., Smith, N. E., Clifford, C. K., and Clifford, A. J. (1980) Comparative Biochem and Physiol., in press
13. Puppione, D. L., Forte, T. M., Nichols, A. V., and Strisower, E. H. (1970) Biochim. Biophys. Acta 292, 392
14. Lindgren, F. T. (1975) in Analysis of Lipids and Lipoproteins (Perkins, E. G., ed) p. 294, American Oil Chemical Society, Champaign, Illinois
15. Allain, C. C., Poon, L. C., Chan, C. S. G., Richmond, W., and Fu, F. C. (1974) Clin. Chem. 20, 470–475
16. Manual of Laboratory Operations, Lipid Research Clinic Program (1974) Lipid and Lipoprotein Analysis Vol. 1, DHHS Publication No. (NIH) 75-628, Department of Health, Education and Welfare, Washington, D.C.
17. Turner, J. D., and Rouser, G. (1970) Anal. Biochem. 28, 437–445
18. Markwell, M. A., Haas, S. M., Biever, L. L., and Tolbert, N. E. (1978) Anal. Biochem. 87, 206–210
19. Weber, K., and Osborn, M. (1975) in The Proteins (Nerurath, R., and Hui, R. L., eds) p. 179, Academic Press, New York
20. Laemmli, U. K. (1975) Nature 227, 680–685
21. Tall, A. R., Small, D. M., Shipley, G. G., and Lees, R. S. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 4940–4942
22. Tall, A. R., Shipley, G. G., and Small, D. M. (1976) J. Biol. Chem. 251, 3749–3755
23. Small, D. M. (1970) in Surface Chemistry of Biological Systems (Blank, M., ed) 55–83, Pergamon Publishing Corp., New York
24. Downing, D. T. (1967) J. Chromatogr. 38, 91–99
25. Jonas, A. (1975) Biochim. Biophys. Acta 373, 471–482
26. Luzatti, V., Tardieu, A., and Agerbeck, L. P. (1979) J. Mol. Biol. 131, 455–473
27. Hamilton, J. A., and Cordes, E. H. (1978) J. Biol. Chem. 253, 5193–5198
28. Yang, S. Y., Gutowsky, J. C., Jacobs, R., King, T. E., Rice, D., and Oldfield, E. (1979) Biochemistry 18, 3257–3267
29. Havel, R. J. (1957) J. Clin. Invest. 36, 848–854
30. Tall, A. R., Green, P. H. R., Glickman, R. M., and Riley, J. W. (1979) J. Clin. Invest. 64, 977–988
31. Redgrave, T. G., and Tall, A. R. (1979) J. Clin. Invest. 64, 182–171
32. Chajek, T., and Eisenberg, S. (1978) J. Clin. Invest. 61, 1654–1655
33. Schaefer, E. J., Jenkins, L. L., and Brewer, H. B. (1978) Biochem. Biophys. Res. Commun. 80, 405
34. Patris, J. R., Goto, A. M., Olivcrona, T., and Eisenberg, S. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 4519–4523
35. Tall, A. R. (1979) Circulation 60, 11–72